The Isolation, Partial Purification, and Preliminary Characterization of a Growth Factor from Chick Embryo Brains

by

Monica Krista Seifried, Honours BSc, Brock University

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BROCK UNIVERSITY
St. Catharines, Ontario

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ABSTRACT

Chick brain growth factor (CBGF) is a mitogen isolated from embryonic chick brains thought to have a potential role as a trophic factor involved in nerve dependent amphibian limb regeneration. In addition, CBGF stimulates ^{3}H-thymidine incorporation in chick embryo brain astrocytes *in vitro*.

In this study, cultured chick embryo brain non-neuronal cells were employed in a bioassay to monitor CBGF activity throughout various stages of its purification. Cell culture and assay conditions were optimized. Non-neuronal cells grew best on collagen-coated culture dishes in complete medium, were most responsive to a growth stimulus [10% fetal bovine serum (FBS)] at the second and third subcultures, and were healthiest when rendered "quiescent" in medium supplemented with 1% FBS. The most effective bioassay conditions consisted of a minimum 14.5 hour "quiescence" time (24 hours was used), a 6 hour "prestimulation" time, and a 24 hour ^{3}H-thymidine labeling time. Four-day subconfluent primary non-neuronal cells consisted of 6.63% GFAP positive cells; as a result cultures were thought to be mainly composed of astroblasts.

CBGF was purified from 18-day chick embryo brains by ultrafiltration through Amicon PM-30 and YM-2 membranes, size exclusion chromatography through a Biogel P6 column, and analytical reverse-phase high-performance liquid chromatography (rp-HPLC). The greatest activity resided in rp-HPLC fraction #7 (10 ng/ml) which was as effective as 10% FBS at stimulating ^{3}H-thymidine incorporation in chick embryo brain non-neuronal cells.

Although other researchers report the isolation of a mitogenic fraction consisting of 5'-GMP from the embryonic chick brain, UV absorbance spectra, rp-HPLC elution profiles, and fast atom bombardment (FAB) mass spectra indicated that CBGF is neither 5'-GMP nor 5'-AMP.

Moreover, commercially available 5'-GMP was inhibitory to ^{3}H-thymidine incorporation in the chick non-neuronal cells, while 5'-AMP had no effect.

Upon treatment with pronase, the biological activity of fraction P6-3 increased; this increase was nearly 30% greater than what would be expected from a simple additive effect of any mitogenic activity of pronase alone together with P6-3 alone. This may suggest the presence of an inhibitor protein. The bioactive component may be a protein protected by a nucleoside/nucleotide or simply a nucleoside/nucleotide acting alone.

While the FAB mass spectrum of rp-HPLC fraction #7 did not reveal molecular weight or sequence information, the ion of highest molecular weight was observed at m/z 1610; this is consistent with previous estimations of CBGF's size.

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INTRODUCTION

Chick brain growth factor (CBGF), a mitogen isolated from 18 day chick embryo brains, is one of several trophic factors proposed to have a role in blastema cell proliferation during the nerve dependent stages of amphibian limb regeneration (Carlone and Rathbone, 1985; Carlone *et al.*, 1987). This supposition is based upon the fact that CBGF (10 ng/ml) has a mitogenic effect on cultured blastema explants from forelimb regenerates of the adult newt *Notophthalmus viridescens* (Carlone *et al.*, 1987) and on mesenchyme-like cells of *Xenopus laevis* limb regenerates (Tsilfidis and Liversage, 1989). Moreover, CBGF activity lacks species specificity, since a fraction isolated from adult newt brains has identical biochemical characteristics as CBGF and is mitogenic for newt blastema cells (Carlone *et al.*, 1987).

CBGF has been purified to apparent homogeneity by ultrafiltration through Amicon PM-30, YM-2, and UM-05 membranes, DEAE ion-exchange chromatography, and reverse-phase high-performance liquid chromatography (rp-HPLC) (Carlone and Rathbone, 1985; Carlone *et al.*, 1987). In addition to its effect on newt blastemas and *Xenopus* regenerates, CBGF also stimulates ^{3}H-thymidine incorporation in 10 day chick embryo brain astrocytes and Swiss mouse 3T3 fibroblasts (Carlone and Rathbone, 1985; Carlone *et al.*, 1988).

The loss of biological activity which occurs when CBGF is incubated with either trypsin (Carlone and Rathbone, 1985) or pronase, in contrast to its stability upon treatment with RNase and DNase (Carlone, unpublished results) suggests that the factor is a peptide. Amino acid analysis has revealed that CBGF is a small polar peptide of 15 amino acids (Carlone *et al.*, 1987). This evidence, in addition to the HPLC retention time, the SDS-polyacrylamide gel electrophoresis mobility (dansylated peptides), and preliminary mass spectrometry data, has approximated the molecular

weight of CBGF at 1,500-1,600 Da (Carlone *et al.*, 1987; Carlone, unpublished results). Other researchers have also isolated astrocyte mitogens from the 18 day chick embryo brain using a slightly different procedure than that used to purify CBGF; the most active fraction was determined to be guanosine 5'-monophosphate (5'-GMP) (Kim *et al.*, 1991) with less potent activities residing in a fraction composed of adenosine 5'-monophosphate (Rathbone *et al.*, 1992a) and other unidentified fractions (Rathbone *et al.*, 1992b).

The bioassay employed to assess CBGF's mitogenic activity at various stages of purification monitors its ability to promote ^{3}H-thymidine incorporation into chick embryo brain astoblasts/astrocytes *in vitro* (Carlone *et al.*, 1987). Because they had not been previously optimized, the first goal of this research was to determine the most effective cell culture conditions and bioassay parameters.

The protocol used to isolate CBGF involved ultrafiltration through a series of Amicon filters of decreasing porosity, with the active fraction being retained on a type UM-05 membrane with a nominal molecular weight cut-off of 500 Da (Carlone *et al.*, 1987). When the manufacture of this membrane was ceased, it was no longer possible to obtain a biologically active retentate from the replacement membrane, YC-05 (Carlone, personal communication). Furthermore, because CBGF was found to be a polar molecule, the inclusion of anion exchange chromatography in the isolation scheme might aid in separation of a more highly purified CBGF. Therefore, the second goal of this thesis was to improve the isolation and purification procedure of CBGF.

As previously stated, earlier studies suggested that CBGF is a small peptide of 1,500-1,600 Da (Carlone *et al.*, 1987). Moreover, other researchers had isolated 5'-GMP and 5'-AMP as astrocyte mitogens from the 18 day chick embryo brain (Kim *et al.*, 1991; Rathbone *et al.*, 1992a). On the other hand, while a CBGF fraction obtained from Rathbone and his

colleagues was found to contain 5'-GMP, it did not stimulate ^{3}H-thymidine incorporation into chick astrocytes; interestingly, preliminary nuclear magnetic resonance (NMR) studies indicated the presence of nucleosides/nuceotides in inactive rp-HPLC fractions and their absence in mitogenic fractions (M.Seifried, honours thesis, 1988). Thus, the third goal of this research was to further characterize CBGF's biochemical properties and to reconcile the contradictory results which have been described.

LITERATURE REVIEW

Growth Factors

The term growth factor has traditionally been used to refer to a peptide or protein that has a potent proliferative effect on certain cell types. *In vitro*, they stimulate DNA synthesis and mitosis by promoting movement through the cell cycle and so have been employed in the maintenance of cultured cells (Brockes, 1984).

However, research on the burgeoning number of factors being found has indicated that they can have far more diverse functions. More than simple mitogens, some growth factors can have an inhibitory rather than stimulatory effect on cell proliferation (Sporn and Roberts, 1988). Moreover, these molecules have also been seen to guide morphogenesis and to induce differentiation in some systems (Mercola and Stiles, 1988). In addition, some factors promote cell survival (Logan, 1990). Growth factors can also affect other aspects of cell function seemingly unrelated to these; for example, epidermal growth factor (EGF) suppresses gastric acid secretion (Gregory, 1975), while interleukin-6 (IL-6) increases secretion of immunoglobulins (Kishimoto and Hirano, 1988). Contributing to the complexity of this subject is the fact that many neuropeptides have been shown to be slow-acting mitogens in addition to their roles as fast-acting neurotransmitters and hormones (Woll and Rozengurt, 1989).

Another complicating factor is that growth factors may be deployed in three distinct delivery mechanisms. In the autocrine method, cells produce growth factors for which they themselves have receptors and to which they respond. Paracrine growth factors, on the other hand, are made in one cell type and act upon their receptors in a nearby, but different cell type. Alternatively, the factor may be distributed through the circulation to distant cells in an endocine manner (Waterfield, 1989).

While growth factors may have various functions and identities, they can also have a broad range of cells for which they are biologically active. An example is provided by transforming growth factor-beta (TGF-β) which is mitogenic for both fibroblasts and osteoblasts, while being anti-proliferative for epithelial cells, T-lymphocytes, and fibroblasts and osteoblasts under other circumstances (Sporn and Roberts, 1988). The presence of other growth factors can profoundly alter the effect a given factor has on its target. This is the case with TGF-β which, in the presence of platelet-derived growth factor (PDGF), stimulates the growth of some fibroblasts *in vitro,* while inhibiting their growth when EGF is present (Roberts *et al.*, 1985). The differentiative state of a cell also appears to influence the actions of growth factors on it (Sporn and Roberts, 1988).

Out of all this confusion, researchers are trying to find a common thread with which to pull all these disparate functions and details together. One simplification has been the discovery that growth factors may be organized into superfamilies, groupings which are based upon nucleotide and amino acid sequence homology in addition to similar receptor-binding activity (Mercola and Stiles, 1988). Thus, for example, the transforming-growth factor-beta family has several members, including: TGF-β_1, TGF-β_2, TGF-$\beta_{1,2}$, inhibin-A, inhibin-B, activin-A, activin AB, the Mullerian-inhibiting substance (MIS) (Mercola and Stiles, 1988) and the bone morphogenic proteins (BMPs - with the exception of BMP-1) (Celeste *et al.*, 1990).

While growth factors from differing superfamilies may lack significant overall sequence similarity, aspects of their three-dimensional structure may be conserved indicating their evolutionary relatedness (McDonald and Hendrickson, 1993). For example, while NGF, TGFβ, PDGF, and their families have distinct biological roles, they belong to a structural superfamily which shares a "cystine knot" motif and a conserved β-strand structure (McDonald and Hendrickson, 1993).

Another feature some growth factors have in common is the action of the receptor to which they bind and begin to mediate their response. The receptors for EGF, PDGF, insulin and insulin-like growth factor I (IGF-I) function as tyrosine-specific protein kinases, phosphorylating protein "second messengers" intracellularly in response to the factor binding to the extracellular domain of the membrane-spanning receptor (Mercola and Stiles, 1988; Bender, 1985).

While growth factors can induce lineage-specific genes, that is one mitogen may stimulate the expression of different genes in differing cell types, they also evoke the expression of the same sets of genes in various tissues. These genes include several proto-oncogenes, among them *c-myc* and *c-fos* which respond to, for example, EGF, PDGF, and nerve growth factor (NGF) (Woodgett, 1989). The products of genes such as *c-myc* and *c-fos* may generate a general growth factor response, the specific nature of which is dependent not only on cell type, but also the differentiative state of the cell. Therefore, it is possible to explain the pleiotropic effects of growth factors using a mechanism in which a common switch is employed by many cell types to yield numerous varying responses (Mercola and Stiles, 1988). Such a common switch points to a conserved signalling scheme that may also be present in the homeotic pattern-forming loci *Notch* in *Drosophila* and *lin-12* in *Caenorhabditis elegans.* This could explain the observed homology between the protein products of these genes and EGF (Bender, 1985).

Another piece in the puzzle is a potential link between differentiation and cell cycle control. With the exception of aspects of embryonic development, proliferation and differentiation seem to be mutually exclusive. Perhaps a simple binary on/off switch to cell division is activated by growth factors to release or arrest the cell cycle and to ultimately control differentiation (Mercola and Stiles, 1988).

One unifying view of the complexities of growth factors has been to

envision them as characters in the alphabet of a cellular signalling language which convey their information via patterns rather than as individuals. Redundancy in the effects of the factors may represent a precaution used to avoid errors in signal transmission (Sporn and Roberts, 1988).

Nerve Dependent Limb Regeneration

A. The events of normal regeneration

Epimorphic limb regeneration is a remarkable ability possessed exclusively by larval and adult urodele amphibians such as newts, salamanders, axolotls, and the tadpole larvae of frogs and toads. Although these are the only vertebrates able to regenerate a limb or tail, all vertebrates are capable of tissue repair to, for example, epithelia, bone, liver, and muscle (Wallace, 1981). Some researchers argue that the cartilaginous spike characteristic of postmetamorphic *Xenopus* regeneration represents a transitional stage between complete epimorphic regeneration and simple tissue repair (Goss and Holt, 1992). Following amputation, normal regeneration ensues, beginning with the rapid migration of epidermal cells to cover the wound within a few hours. This migration continues, and the wound epithelium thickens, largely without concomittant local mitosis, to form the apical epidermal cap (Repesh and Oberpriller, 1980). The specialized wound epithelium of the regenerate differs from that encountered during normal wound healing since it lacks a basement membrane, thereby coming into direct contact with underlying tissues; in fact, without this immediate contact, regeneration is prevented. Underneath this epithelial jacket, undifferentiated mesenchymal progenitor cells migrate from the stump mesoderm to form a local growth zone known as the blastema (Singer, 1978; Wallace, 1981; Brockes, 1987a).

The issue as to whether blastema cells originate from reserve cells (Cameron *et al.*, 1986) or by dedifferentiation from various adult tissues (Casimir *et al.*, 1988) remains unresolved. However, recent findings demonstrate that multinucleate myotubes implanted into the limb blastema are induced to dedifferentiate into mononucleate cells which proliferate; limited evidence indicates that these cells subsequently redifferentiated, with some undergoing metaplasia (Lo *et al.*, 1993). The epidermal cells do not appear to contribute to the cells of the blastema and the reverse apparently also holds true (Chalkley, 1954; Hay and Fischman, 1961; O'Steen and Walker, 1961). The distally migrating blastema cells commence cell division 3-5 days after amputation and continue rapid proliferation for 2-3 weeks giving rise to a protruding cone of cells. The cells subsequently form into a flattened palette shape and begin to display the characteristic protrusions that further elongate to become the digits (Wallace, 1981). During this period, the blastema redifferentiates in a proximal to distal sequence, and undergoes morphogenesis, thereby reconstructing the necessary cartilage, muscle, and connective tissues of the regenerate limbs. The wound epithelium is thought to have a role in cell proliferation, the maintenance of a gradient of mesenchyme differentiation, and the direction of regenerate outgrowth (Wallace, 1981). In the adult newt, the final functional and normally sized replacement is completed after approximately five weeks, virtually irrespective of where along the proximal-distal axis amputation occurred (Brockes, 1987a).

B. The nerve dependence of regeneration

Regeneration, and in particular, early blastema cell proliferation, is under the influence of peripheral nerves having axons present at the amputation plane (Todd, 1823; Singer, 1952; 1978; Brockes, 1984; 1987a). If the limb is denervated prior to amputation, the early non-proliferative

events of regeneration take place: the wound epidermis forms and the first blastema cells arise from the mesoderm; however, the usual onset of blastema cell division is prevented, as evidenced by the eventual decrease in both the ^{3}H-thymidine labelling index and mitotic index (Singer and Craven, 1948; Maden, 1978). The synthesis of RNA and protein are also curtailed. Thus, it appears that denervation dramatically retards cell cycle activity (Wallace,1981).

Although it is debated as to whether denervation causes blastema cells to block in G_1 or G_2 of the cell cycle, recent evidence tends to support the hypothesis of a blockage in G_1 (Oudkhir *et al.*, 1985; Tassava *et al.*, 1987; Goldhamer and Tassava, 1987) with some cells prematurely exiting the cell cycle into the G_0 phase (Boilly *et al.*, 1986). The "punctuated" cycling phenomenon, suggested by Tomlinson *et al.* (1985), proposes the existence of at least two blastema cell subpopulations, an actively cycling population and a transiently quiescent population, which compose the proliferative fraction of the blastema; nerves appear to act primarily in G_1 by controlling the entry of transiently quiescent cells into the actively cycling population (Goldhamer and Tassava, 1987). Denervated and amputated axolotl limbs exhibit reductions in the size of the proliferative and actively cycling fractions of the blastema cell population with prolonged denervation, while innervated limbs display an increase in the actively cycling population, at the expense of transiently quiescent cells, during the period of rapid blastemal growth (Tomlinson and Barger, 1987).

Immediately following denervation, there is an initial burst of DNA, RNA, and protein syntheses, as well as an increase in the number of mitoses. This observation, in combination with the later effects of denervation, suggest that the nerves release a trophic factor which stimulates the cell cycle and promotes blastema cell proliferation (Singer, 1974; 1978; Brockes, 1984; 1987a). Cutting of the nerve, as in denervation, apparently causes an initial rapid discharge of the trophic

substance from the degenerating axons, after which no replenishing source of the factor exists. Consequently, the blastema does not grow, the subsequent events of regeneration do not take place, and formation of the regenerate ceases.

When denervation is postponed for up to approximately 14 days following amputation, blastema cell proliferation drops markedly and regeneration stops, with the blastema being converted into fibrocellular tissue and cartilage (Singer, 1978). After this point, however, when the blastema has reached the palette stage, a critical mass of blastema cells has formed since denervation causes a less severe decrease in the mitotic index and a normal, albeit smaller, limb is formed. Therefore, while the nerve and its putative trophic factor control progenitor cell proliferation during the first two weeks following amputation, they have no bearing on the nature of the structure formed (Singer, 1978; Wallace, 1981).

Extensive studies of newt forelimb innervation density and regeneration were conducted by Singer (1952; 1974). He found that the forelimb of the adult newt is innervated by the third, fourth, and fifth spinal nerves which merge at the brachial plexus before dividing and entering the limb; the forelimb is also innervated by a minor sympathetic component. The density and type of innervation, be it motor or sensory, may be varied by cutting the distal axons and allowing them to degenerate, leaving the denervated Schwann cell sheaths in their wake. In this way, it was possible to demonstrate that there exists a quantitative relationship between the density of innervation at the amputation surface and the frequency of regeneration (Singer, 1947). However, Singer's hypothesized threshold innervation density absolutely required for regeneration to take place does not seem to be a valid assumption since other researchers have found no evidence for such a limiting value (Karczmar, 1946; Wallace, 1981); in fact, regeneration can occur even in the total absence of nerves in the special circumstance of the aneurogenic

limb (to be described in detail later) (Yntema, 1959a,b). Moreover, not only is the frequency of regeneration directly proportional to the available nerve supply, but it is independent of whether the innervation is exclusively of a motor or a sensory nature (Sidman and Singer, 1960). In addition, regeneration can take place as long as the axons at the cut site remain intact with their cell bodies; a complete pathway to the central nervous system is not necessary. This further seems to indicate the involvement of an axonally transmitted factor, which does not seem to require involvement of Schwann and connective tissue sheath cells (Wallace, 1981). However, nerve augmentation by implantation of dorsal root ganglia into normally innervated newt blastemas does not cause an increase in cell cycle activity, indicating that levels of the nerve's trophic factor are not quantitatively limiting and are not responsible for the relatively small actively cycling cell population of the adult newt blastema (Goldhamer *et al.*, 1992).

Interestingly, previously injured axons regenerate more rapidly than than those only subjected to a testing lesion and concomitantly cause accelerated limb regeneration *in vivo* (Maier *et al.*, 1984); regrowing nerves also demonstrate enhanced mitogenicity for newt blastemas *in vitro* (Boilly and Bauduin, 1988).

C. A nerve dependent subpopulation of blastema cells

The question as to whether the undifferentiated mesenchymous cells of the blastema are an homogeneous population or whether they are composed of molecularly disparate cells has been addressed by Brockes and his colleagues (Brockes, 1984; 1987a; 1991). They immunized mice with early forelimb blastemas from the newt and screened the antibody-secreting clones by immunofluorescence on sections of the early limb regenerate; the 22/18 antibody was the only one isolated which

differentiated between blastema cells and those of the unamputated limb, preferentially labelling the progenitor cells of the regenerate (Kintner and Brockes, 1984; 1985).

In regenerating tissue, the 22/18 antigen is a cytoskeletal component, apparently a conformational determinant on intermediate filaments as evidenced by the intracellular, filamentous staining pattern seen in 22/18 immunoreactive cells (Fekete *et al.*, 1987; Ferretti and Brockes, 1990). The expression of the 22/18 antigen begins within 36-48 hours after amputation and is initially confined to non-neural, non-muscle interstitial cells of a bipolar or dendritic morphology; this cell type is located throughout injured tissues such as nerve, muscle, and dermis (Gordon and Brockes, 1988). These cells may represent a type of reserve cell that makes an early contribution to the blastema. The 22/18 + cells sometimes observed in intact healthy newt limbs had a similar morphology and interstitial location as that seen for regenerating tissue, but displayed a granular, as opposed to filamentous staining pattern. This complementary distribution points to a potential role for granular-staining 22/18 + cells of the normal limb as precursors to the filamentous-staining 22/18 + cells of regenerating tissue (Gordon and Brockes, 1988). The early appearance of 22/18 + cells occurs prior to DNA synthesis and proliferation of the blastema cells, indicating that expression of the antigen is not induced by mitosis. Four to five days post-amputation, expression of the 22/18 antigen is also observed in Schwann cells of the nerve sheath, but is not seen in axons or in fibroblasts (Kintner and Brockes, 1984; 1985). Anatomical studies have also suggested that Schwann cells may contribute to the blastema following dedifferentiation (Brockes, 1984). After about twelve days, it is clear that muscle cells also contribute to the blastema, since cells that stain with both the 22/18 and a myofiber-specific antibody can be seen (Kintner and Brockes, 1984). Incidentally, such double-labelled cells may represent intermediates in dedifferentiation,

temporarily displaying properties of differentiated parent cells and blastema cells. Cells with this staining pattern reappear about three weeks after amputation as blastema cells begin to redifferentiate into muscle cells (Brockes, 1984). More evidence for dedifferentiation and metaplasia comes from studies using DNA hypomethylations as lineage markers to follow cells fates during limb regeneration (Casimir *et al.*, 1988); such investigations have suggested that cells from muscle and connective tissue lineages contribute to the cartilage of the regenerate.

In the two weeks following amputation, approximately 80% of blastema cells react with the 22/18 antibody. However, in the following week, 14-21 days after amputation, the differentiating blastema cells progressively begin to lose reactivity with the antibody (Kintner and Brockes, 1985). The fully differentiated regenerate, like the normal unamputated limb, is essentially 22/18 -. Moreover, 22/18 expression is not a transient response to injury, but a stable one, as evidenced by its continued expression even when regeneration is blocked *in vivo* (Fekete *et al.*, 1987; Gordon and Brockes, 1988). Expression of this antigen is also stable in dissociated blastema cell cultures (Ferretti and Brockes, 1988).

Circumstances less dramatic than amputation are sufficient to elicit the production of the 22/18 antigen. Local injury, such as cutting the brachial nerve or bruising the limb, causes its expression, suggesting that this event reflects the activation of mesenchymal cells to participate in both repair and regeneration processes (Gordon and Brockes, 1988). These *in vivo* observations extend to *in vitro* conditions (Ferretti and Brockes, 1988). The antigen 22/18 is present in cultures of normal limb, heart, and liver tissues, but is virtually absent from such tissue sections. The preparation of cultures like these, with its necessary disruption of tissue integrity and enzymatic dissociation of cells is tantamount to injury, like that produced in the *in vivo* situation. This is further emphasized by the fact that although the number of 22/18 + cells is small in dense cultures, it

increases when the cells are removed, or wounded, and replated at a lower density (Ferretti and Brockes, 1988).

Not only does the 22/18 antibody identify injured and regenerating tissues, it acts as a marker for a subpopulation of newt blastemal cells that is persistently dependent on the nerve for proliferation (Kintner and Brockes, 1985). When assayed two days after denervation, early (13 day - majority of 22/18 + cells) and late (25 day - minority of 22/18 + cells) newt forelimb blastemas displayed a seven-fold decrease in the tritiated thymidine labelling index of 22/18 + cells. The 22/18 - populations was similarly affected, but to a lesser extent, particularly in the late blastema. Any potential candidate for the nerve-released trophic factor must stimulate division of 22/18 + blastema cells since their proliferation is nerve-dependent (Brockes, 1984; 1987a). Just as the initial complement of blastema cells forms even in the absence of the nerve and does not undergo mitosis, the expression of the 22/18 antigen does not require the presence of the nerve or that cell division occur (Kintner and Brockes, 1985; Gordon and Brockes, 1988). This is consistent with 22/18 expression in the absence of the nerve *in vitro* (Ferretti and Brockes, 1988). However, the nerve and its trophic factor are required for blastema cell proliferation and specifically those blastema cells which are 22/18 +. Thus, the signals governing the induction of 22/18 expression differ from the mitogenic signals leading to 22/18 cell division. A potential role for Schwann cells and a factor which these cells may elaborate has been suggested in the induction of 22/18 expression (Fekete *et al.*, 1987; Gordon and Brockes, 1988). The presence of at least two blastema cell populations, one that is 22/18 + and another that is 22/18 -, which are differentially affected by denervation suggests that two systems of growth control may also exist: one is absolutely dependent on the nerve, while the second is not (Brockes, 1987a).

This second system of control is also intimated in studies of 22/18

expression in the limb bud of the newt embryo (Fekete and Brockes, 1987a). The antigen is not apparent in the flank mesenchyme before outgrowth of the bud, and is seen in less than 1% of mesenchymal cells once the limb bud develops. Thus, although various similarities between the initial development of a limb in the embryo and regeneration of an amputated limb in the adult exist, the 22/18 antibody specifically marks cells involved in regeneration and not early development. This changes once the developing limb becomes innervated, once again showing that the 22/18 antigen is a marker for cells whose proliferation is nerve dependent. The experimental evidence for this comes from research examining the 22/18 reactivity in the amputated limb bud at a variety of developmental stages (Fekete and Brockes, 1987a). Regardless of the stage at which amputation occurs, the bud or developing limb regenerates. When amputation takes place early, before nerves have reached the amputation plane, no significant accumulation of 22/18 expression is observed. In contrast, 22/18 + cells are seen to populate the blastema when amputation is performed after the two-digit stage and nerve arrival. These observations are not a function of embryonic development, but of the stage of limb development. This is illustrated by the fact that at a certain stage, the amputated forelimb will yield a 22/18 + blastema, while the amputated hindlimb, whose development is retarded when compared to that of the forelimb, will produce a 22/18 - blastema. Moreover, the 22/18 expressing cells are seen in close proximity to the nerve. Evidently, the change, during regeneration of the developing limb bud, from a 22/18 - to a 22/18 + blastema is correlated with the appearance of axons and Schwann cells at the amputation plane (Brockes, 1987a; Fekete and Brockes, 1987b). As was suggested in the case of regeneration, it seems possible that a second system of growth control, other than that provided by the nerve, is acting on the 22/18 - negative cell population in the normal developing limb bud. In some way, the process of innervation has

the consequence of negating the effectiveness of this system, leaving the blastema cells dependent on the nerve (Brockes, 1987a; Ferretti and Brockes, 1991). This may take place by altering the responsiveness of cells already present in the limb prior to innervation, by introducing a new migratory nerve-dependent population of cells, such as Schwann cells, or by employing a combination of these two scenarios (Fekete and Brockes, 1987b).

D. The aneurogenic limb

A challenge to the theory that the nerve produces and releases a factor which promotes blastema cell proliferation and permits regeneration (Singer, 1952) has come from studies examining the phenomenon of regeneration of the aneurogenic limb. Yntema (1959a; b) first described the procedure that led to the formation of aneurogenic urodele forelimbs. When they have reached the head process stage, prior to forelimb emergence, two *Ambystoma* embryos are fused in parabiosis with the result that the two share a common yolk supply and, in time, a common circulation. One of the partners undergoes surgery to remove the ectodermal placodes and much of the neural tube; the unoperated partner sustains the development of the other. The forelimb bud of the operated embryo proceeds to develop normally in the absence of a nerve supply and at the same rate as that of the other member of the pair; without the trophic support of the nerve, however, the muscle later begins to degenerate (Tweedle *et al.*, 1974).

The remarkable thing about the aneurogenic limb is that, upon amputation, it forms a blastema whose cells proliferate and form a regenerate despite the absence of nerves (Yntema, 1959a). This line of investigation was furthered by Thornton and Thornton (1970) who transplanted the aneurogenic limbs to normal larvae which had had their

forelimbs removed. After approximately ten days, the brachial nerves of the host have innervated the transplant. If the limb is denervated at this point and then amputated, the limb will regenerate as usual. However, between days 10-13 after transplantation this changes and regeneration becomes nerve dependent. Furthermore, they found that transplanted limbs that were left for 33 days and then kept denervated for another 30 days regained their ability to regenerate in the absence of nerves in 50% of the animals tested. This situation is contradictory to that seen for the normal limb, which, even if maintained in a denervated state for 7-8 weeks does not lose its nerve-dependence upon amputation and thus does not regenerate (Liversage and McLaughlin, 1983; Scadding, 1984).

The different circumstances under which the normal and aneurogenic limbs regenerate extends to a detectable difference at the molecular level. Therefore, unlike the normally innervated limb, the aneurogenic limb regenerates with a 22/18 - blastema; it is predicted that once the limb is innervated and under the control of nerve dependent regeneration, it would regenerate with a 22/18 + blastema (Fekete and Brockes, 1988; Ferretti and Brockes, 1991).

The situation seen for the aneurogenic limb resembles that seen for the preinnervated limb bud and, once again, a second system of growth control, other than that provided by the nerve, is suggested. Evidently, a change from nerve-independent to nerve-dependent growth control has taken place in both of these cases and the 22/18 antibody serves as a marker for this transition; a nerve-independent system of growth control also seems to be functioning in the 22/18 - cells of the normally innervated blastema, particularly at later stages of regeneration (Fekete and Brockes, 1987b). Although the 22/18 antibody detects a change in the cells at the time of amputation in the presence of nerves, it is not known whether this effect is a consequence of an earlier one that occurred upon innervation (Fekete and Brockes, 1988). Attempts to explain how nerves

control regeneration have led to the proposal of at least two hypotheses (Singer, 1978; Wallace, 1981; Brockes, 1987a). One possibility is that the non-neural cells of the developing limb produce other growth factors that stimulate blastema cells of the regenerate to divide, but the presence of a nerve supply suppresses this production and causes proliferation to become nerve-dependent. Singer (1978) has suggested that the same trophic activity exhibited by the neuron is common to all cells, but that the great excess of this agent made in the nerve spills over to the surrounding cells, thereby diminishing their production of the factor. Another alternative is that nerves or their trophic material alter the limb cells in such a manner that they are no longer responsive to growth factors other than those manufactured by the nerve (Brockes, 1987a). Either scenario has the limb becoming "addicted" to the nerve as its source of the mitogen (Brockes, 1984). The results of experiments employing the 22/18 antibody support the latter hypothesis (Fekete and Brockes, 1987b; 1988).

Brockes and his colleagues propose a role not only for nerve axons in the establishment of nerve-dependent limb regeneration, but also for blastema cell precursors arising at the amputation site and particularly for Schwann cells (Brockes, 1984; 1987a; Fekete and Brockes, 1987b; 1988). When innervation takes place, it is not merely axons which invade limb tissue, but also their associated Schwann cells. Upon amputation of the normally innervated limb, axons transiently retract from the amputation plane, leaving behind a small segment of denervated Schwann cells. It appears then that the common thread tieing all cases of a 22/18 + nerve-dependent blastema together is amputation in the presence of denervated Schwann cells (Brockes, 1987a). This contention is supported by a number of observations. For example, regeneration is inhibited in irradiated limb stumps even though axonal growth continues unhindered (Wallace and Wallace, 1973); loss of regenerative capacity may be a result of the suppression of Schwann cell division (Maden, 1977). Moreover, Schwann

cells have been shown to contribute to the cells of the blastema and express the 22/18 antigen in response to injury and amputation (Kintner and Brockes, 1984; 1985; Gordon and Brockes, 1988). The presence of 22/18 + Schwann and interstitial cells upon amputation of the denervated limb adds further credibility to the notion that it is denervated Schwann cells at the amputation plane, and not axons alone, that give rise to a 22/18 + nerve-dependent blastema (Kintner and Brockes, 1985). On the other hand, Schwann cells are obviously unable to contribute to cells forming the blastema of a regenerating preinnervated limb bud or aneurogenic limb and so a 22/18 - nerve-independent blastema results (Brockes, 1984; 1987a). Furthermore, the demonstrated growth factor requirements of Schwann cells *in vitro* can be supplied by the mitogen glial growth factor, an activity residing in the innervated, but not denervated newt blastema (Brockes and Kintner, 1986). By early removal of the neural crest, researchers hope to acquire newt limbs containing axons, but lacking Schwann cells; they anticipate that amputation of such limbs will give 22/18 - blastemas (Brockes, 1987a; Fekete and Brockes, 1988).

E. The nature of the nerve's trophic factor

Ever since Singer (1943) first proposed his trophic theory in which nerves elaborate a factor which promotes blastema cell proliferation and thereby permits regeneration to take place, numerous researchers have sought to identify the molecule responsible (Singer, 1978; Wallace, 1981; Brockes, 1984; Brockes and Kintner, 1986; Liversage, 1987). The causative agent is sometimes referred to as the neurotrophic factor, however this term is confusing since it has disparate meanings. In the present context, the molecule being discussed has the characteristics of a mitogenic growth factor. The term neurotrophic factor is used to describe

the substances released by nerve targets to ensure the survival of those developing neurons which are able to secure the molecule on their receptors (Barde, 1989); this label is also given to the factors involved in the various effects nerves have on muscle which are independent of cell division (Brockes, 1984).

Although the nature of the nerve's growth stimulus is unknown, it is unlikely that electrical impulses are involved (Wallace, 1981). This conclusion is based upon the fact that both sensory and motor neurons can promote regeneration even though their axons typically propagate impulses in opposite directions. This, in turn, suggests that repeated activation or use of a nerve's target tissue is not necessary to mediate the trophic response, an assumption supported by the observation that normal regeneration can occur even under conditions of prolonged paralysis induced by botulinum toxin (Drachman and Singer, 1971).

Various pieces of evidence point to a soluble, diffusible factor as being the nerve's trophic agent in limb regeneration (Singer, 1978; Brockes, 1984; Carlone and Mescher, 1985). Perhaps the first indication of the chemical nature of this factor came from the studies of Lebowitz and Singer (1970) in which they observed that the infusion of aqueous extracts of spinal ganglia into denervated blastemas *in vivo* caused an increase in the rate of protein synthesis to control levels. This effect could be mimicked by injection of aqueous extracts of central nervous tissue and could be prevented by inactivation of the factor by heat or trypsin treatment (Singer *et al.*, 1976); these findings more specifically implicate a soluble protein in the role as the nerve's factor. Moreover, when the regenerate is physically isolated in culture from sensory ganglionic neurons by a filter of very low porosity (0.45 μm or 0.05 μm) (Globus and Vethamany-Globus, 1977; Tomlinson *et al.*, 1981), the blastema cells divide. Since cytoplasmic processes from both the nerves and the regenerate are too large to penetrate the filter, only a chemical released by

the nerve is small enough to pass through it. The factor must also be diffusible since the highest mitotic index is observed in those blastema cells located closest to the nervous tissue (Globus and Vethamany-Globus, 1977). In addition to demonstrating the chemical nature of the nerve's trophic molecule, these experiments bring to light another important feature of this system: direct physical contact between neuronal processes and blastemal cells is not required for the induction of a mitogenic effect (Globus and Vethamany-Globus, 1985). This is evidently the case despite early suggestions to the contrary (Salpeter, 1965; Tassava and Olsen, 1985).

Evidence for the supposition that the trophic factor is produced in the neuronal cell body and delivered to the target cells via the axonal ending (Singer, 1978) is provided by studies showing ^{3}H-leucine-labelled protein transport during axonal regeneration in larval and adult limbs (Scadding and Liversage, 1979; Lentz, 1972). Wallace (1981) compiled data from various laboratories to conclude that in order for RNA synthesis in the blastema to decline within six to twelve hours following denervation, the trophic factor must be depleted in a manner consistent with fast axonal transport (Singer and Caston, 1972).

Researchers attempting to determine the chemical agent responsible for the nerve's mitogenic influence on the regeneration blastema have employed both *in vivo* and *in vitro* methods of testing (Wallace, 1981; Globus and Vethamany-Globus, 1985; Carlone and Mescher, 1985). Initial strategies involved an attempt to replace the trophic factor missing from amputated denervated limbs by injecting or infusing various substances or extracts *in vivo*, thereby hoping to stimulate complete regeneration. Although a limited amount of growth was observed in some cases (Kamrin and Singer, 1959), these types of experiments have not been fruitful. Other studies which anticipated excessive regeneration or its inhibition upon the injection of test solutions were also, for the most part,

unsuccessful (Wallace, 1981). The downfall of this type of assay is the difficulty in mimicking the postulated prolonged delivery of the trophic factor under laboratory conditions (Singer, 1978). For this reason, a more successful technique has been to measure the effects of test chemicals and extracts on the rates of macromolecular synthesis and mitosis, systems operating on a much shorter time scale than full regeneration. This method was used by Lebowitz and Singer (1970) in studies demonstrating the ability of nervous tissue extracts to overcome the decline in protein synthesis following denervation of the amputated newt limb. Goldhamer *et al* (1992) have suggested that maintaining cell cycle activity in short-term *in vivo* experiments with trophic factor candidates may be a more realistic and productive strategy than restimulation bioassays. Furthermore, researchers have applied their ability to maintain explanted amphibian blastemas in organ culture to the development of *in vitro* bioassays that test the effectiveness of known chemicals and extracts on protein and DNA synthesis as well as mitosis (Globus and Vethamany-Globus, 1977; 1985; Choo *et al.*, 1978; Carlone and Foret, 1979). The *in vitro* system is advantageous since it is convenient, allows the blastema to be isolated from factors delivered by way of the circulatory system, solves the problem of regrowth of nerve fibers into denervated regenerates *in vivo*, and permits experimental flexibility. Even though some may argue the validity of extrapolating *in vitro* results to *in vivo* circumstances, studies have shown that qualitatively and quantitatively similar changes in the rate of macromolecular synthesis occur in both cases (Choo *et al.*, 1978; Mescher and Loh, 1981; Singer, 1974). Preliminary investigations of candidates for the nerve's trophic factor are often undertaken by testing their mitogenic effect on cultured cells, particularly cell lines such as murine 3T3 fibroblasts (Brockes, 1984). Maier and Miller (1992) recently demonstrated that dissociated cultures of newt limb blastema cells were also suitable for testing blastema cell proliferation in response to a crude

newt brain extract.

When testing a trophic factor for its potential role in nerve dependent limb regeneration, Brockes (1984) has outlined four criteria which it must meet: 1) the factor must be present in the blastema, 2) its level must decrease following denervation of the blastema, 3) it must stimulate cell division of the blastema cell population which is dependent on the nerve for proliferation (namely, 22/18 + cells of the blastema), and 4) an antibody against the factor should block its biological activity and prevent the mitogenic effects of the nerve, thereby mimicking denervation. While no single molecule has yet satisfied all four requirements, a growing list of substances are potential candidates and have already met some of these criteria.

The discovery that nerve impulses are mediated by the secretion and transport of chemicals known as neurotransmitters at the neuronal synapse led researchers to investigate the possibility that these molecules might have a dual role, functioning also as the nerve's trophic factor which participates in limb regeneration (Singer, 1978; Carlone and Mescher, 1985). Various experiments have conclusively eliminated acetylcholine as a candidate. In the aforementioned investigation by Drachman and Singer (1971), botulinum toxin produced its paralyzing effects by blocking the release of acetylcholine from nerve endings and yet regeneration proceeded unhindered. This is supported by *in vivo* and *in vitro* studies in which acetylcholine was administered to denervated urodele limbs or cultured limb regenerates, but was found to elicit no mitogenic response (Singer, 1960; Foret and Babich, 1973); in fact, this neurotransmitter reduced protein synthesis in cultured blastemas (Foret and Babich, 1973). Furthermore, it is also unlikely that norepinephrine is the elusive factor since regeneration normally takes place in the absence of sympathetic neurons which release this neurotransmitter (Singer, 1943). Like acetylcholine, norepinephrine is an ineffective proliferative agent for both

denervated and cultured blastemas (Rathbone *et al.*, 1980).

The results of experiments conducted using the neuropeptide substance P are more ambiguous. Globus *et al.* (1983) demonstrated that this undecapeptide was mitogenic for cultured blastemas at picomolar concentrations. Further evidence for a possible role as the nerve trophic factor involved in blastema cell proliferation *in vivo* can be seen in continued studies which illustrated that antiserum to substance P caused a suppression of the mitotic index of blastemas cocultured with dorsal root ganglia; this negative effect on cell division decreased with progressively lower antibody concentration (Globus *et al.*, 1983). This research group has also found that immunofluorescent substance P is consistently localized in the periphery of epidermal cells of the newt limb blastema (Globus and Vethamany-Globus, 1985) and that this immunofluorescence gradually diminishes following denervation of the limb (Globus *et al.*, 1991). Moreover, the discovery that substance P is present at high concentration in the synaptosomal fraction of subcellular fractions of rat hypothalmic tissue (Whittaker *et al.*, 1964) coincides with the finding that the desired trophic factor resides within synaptosomes (Singer *et al.*, 1976). It has been suggested that one mechanism whereby substance P may mediate its effect on the blastema cell proliferation is by stimulating the inositol phospholipid signaling pathway (Globus *et al.*, 1991). However, while this neurotransmitter has been found to be abundant in the hypothalamus, sensory neurons, and sympathetic nerve fibres (Jessel, 1981), it has not been detected in motor neurons. A role for substance P as the sought-after blastema mitogen is suspect in light of the fact that motor neurons alone can promote regeneration, even though they are less effective than a sensory nerve supply (Singer, 1952). While most neurotransmitters have been eliminated as candidates, the arguments supporting a role for substance P in blastema cell proliferation during limb regeneration are not entirely convincing either. Thus, the identity of the nerve's trophic agent

remains to be elucidated. Singer (1978) proposed that not merely one factor exists, but several of these chemicals contribute to an ongoing molecular "conversation", with target cells selecting the molecular "words" which they recognize. Such a scheme allows for the presence of and encourages the quest for numerous relevant blastema mitogens.

The experiments of Lebowitz and Singer (1970) were a pioneering attempt to isolate the trophic factor by making a crude extract from newt brachial nerves. As previously mentioned, the extract promoted an increase in protein synthesis when infused into denervated blastema *in vivo*. Singer and his colleagues subsequently found the activity was also present in homogenates of central nervous tissue which were also found to increase DNA synthesis in denervated regenerates (Singer, 1974; Singer *et al.*, 1976). Other researchers have noted that spinal chord extracts from axolotls with regenerating late bud stage limbs are more potent than such extracts from control unamputated animals, causing an increase in the mitotic index of cultured blastema cells (Boilly and Albert, 1988).

The mitogenic activity was reasoned to be a protein based on the fact that it was heat labile and trypsin sensitive. Moreover, the active component remained stable when frozen for at least two months, was thought to be basic, and was estimated to have a molecular weight of less than 300,000 Da. Because synaptosomes derived from frog brain also appeared to possess the trophic factor, this mitogen lacks species specificity (Singer *et al.*, 1976).

This line of investigation was furthered by Choo *et al.* (1978) who determined that aqueous extracts from the brains of both adult newts and chick embryos could prevent the alterations in protein synthesis (initial rapid increase followed by a decrease) observed in cultured blastemas. The presence of similar trophic activities in both the newt and chick brains parallels the finding of Singer and his colleagues that the factor lacks species specificity (Choo *et al.*, 1978; Singer *et al.*, 1976). Furthermore, the

chick-derived mitogen was also heat labile, protease and trypsin sensitive, but ribonuclease insensitive, confirming Singer's earlier suggestion that the factor is a peptide or protein. Continued purification of the chick brain factor indicated that the crude extract was composed of basic proteins which could be resolved into slow and fast running components based on their electrophoretic behavior on a polyacrylamide gel; each of these regions was further separated into five common proteins having molecular weights ranging from 13,500 to 47,000 Da (Choo *et al.*, 1981). In contrast to the isolation made by Singer *et al.* (1976), the activity found in the chick brain supernatants is unstable to freezing.

A number of more well-characterized growth factors derived from neural tissues have been implicated as candidates for the regenerating urodele limb's nerve-released trophic agent. In particular, researchers, like Choo *et al.* (1978), have found it advantageous to examine the relevance of neural growth factors from higher vertebrates in limb regeneration since the small size of the amphibian nervous system has proved unsuitable for the extensive purification of a blastema mitogen. For example, a mitogen from chick embryo brains that differs from those reported by Choo *et al.* (1981) has also been partially characterized. Termed chick-brain-derived growth factor (CBGF), it stimulates DNA synthesis in cultured newt limb regeneration blastemas. It was originally characterized as a trypsin sensitive basic peptide of low molecular weight (approximately 1500 Da) that has also been isolated from adult chick and newt brains (Carlone and Rathbone, 1985; Carlone *et al.*, 1987). This molecule is stable to heating, freezing, and treatment with RNase, DNase (Carlone, unpublished results), and the disulfide bond reducing agent β-mercaptoethanol (Carlone and Rathbone, 1985). What distinguishes this trophic agent from those of Singer *et al.* (1976) and Choo *et al.* (1981) are its small size and heat stability. Moreover, CBGF appears to be chemically distinct from other low molecular weight blastema mitogens such as

substance P and epidermal growth factor (Globus *et al.*, 1983; Mescher and Loh, 1981). Detailed discussion of CBGF can be found in the following section.

Hydra head activator (HHA) is a neuropeptide (1,142 Da.) originally isolated from the freshwater coelenterate *Hydra* where it controls head-specific growth and differentiation processes (Schaller and Bodenmüller, 1981). Since then, HHA has been found in human and bovine brain, as well as rat intestine (Bodenmüller and Schaller, 1981). In addition to its role as a morphogen in *Hydra*, this neuropeptide acts as an autocrine growth factor for neuroblastoma cell lines (Schaller *et al.*, 1989) and promotes DNA, RNA, and protein synthesis in cultured chick embryo brain cells (Kajiwara and Sato, 1986). Recently, HHA expression was demonstrated in the the adult newt brain, eye, intestine, and regenerating blastema. However, in the blastema, HHA was localized to the apical epidermal layer, with only very low levels evident in the mesenchyme, suggesting that it is synthesized in the wound epithelium (Fuentes *et al.*, 1993). Furthermore, only limited blastema growth was apparent in a small number of denervated limb regenerates implanted with HHA-soaked beads (Fuentes *et al.*,1993).

Transferrin, a common growth factor for cells *in vitro* (Barnes and Sato, 1980) and a demonstrated myoblast mitogen (Stamatos *et al.*, 1983) has also been shown to exert a trophic influence on the denervated newt forelimb blastema by increasing DNA synthesis and the mitotic index both *in vivo* and *in vitro* (Mescher and Munaim, 1984; Carlone and Mescher, 1985; Mescher and Kiffmeyer, 1992); in fact, transferrin has been found to be as effective as neural extract or serum in effecting blastema cell proliferation *in vitro* (Mescher and Munaim, 1984; Albert and Boilly, 1988). Transferrin, the major iron transport protein in vertebrate blood, can also be purified from central and peripheral nervous tissue (Stamatos *et al.*, 1983; Popiela *et al.*, 1984). Mescher and Munaim (1984) found that

the mitogenic effect exhibited a bell-shaped dose response and argued that transferrin was involved in the delivery of iron to blastema cells via specific receptors on their surface. Interestingly, the blastema growth-promoting activity found in neural extracts can be removed by dialysis with an iron-chelating agent and completely restored by readdition of iron, strongly suggesting a role for transferrin during blastema cell proliferation (Munaim and Mescher, 1986). Recently, it was demonstrated that transferrin concentrations increase dramatically in the sciatic nerves of adult axolotls during limb regeneration and was found to be distributed in both axons and Schwann cells; moreover, transferrin is carried by fast anterograde axonal transport and released from distal regenerating axons (Kiffmeyer *et al.*, 1991). These authors also demonstrated that axotomy causes a reduction in the transferrin concentration of early bud stage blastemas, but does not result in its complete depletion (Kiffmeyer *et al.*, 1991); this residual transferrin may be supplied by the vasculature. To facilitate the phase of rapid proliferation required during the largely avascular early stages of regeneration, these researchers reasoned that nerves would supplement the supply of transferrin provided to the blastema vascularly (Kiffmeyer *et al.*, 1991; Mescher, 1992). This situation mirrors Singer's proposal; under the conditions of nerve-dependent limb regeneration, the nerves release large quantities of a trophic factor which is already ubiquitously available to cells, but in much smaller amounts (Singer, 1978).

Acidic and basic fibroblast growth factors (aFGF and bFGF), members of the family of fibroblast or heparin-binding growth factors, were initially isolated from bovine pituitary and brain as potent mitogenic agents for 3T3 fibroblasts *in vitro* (Gospodarowicz, 1974; 1987). Since that time, members of the FGF family have been found to affect the proliferation and differentiation of a wide variety of cell types of mesodermal and neuroectodermal origin, with some of the FGFs demonstrating transforming

potential (Baird and Walicke, 1989; Burgess and Maciag, 1989); FGF has also been implicated as having a role in blastema cell proliferation during amphibian limb regeneration (Gospodarowicz, 1990).

Early *in vivo* studies demonstrated the ability of FGF to promote the resumption of mitotic activity in mesenchymal cells when infused into denervated newt forelimb blastemas (Mescher and Gospodarowicz, 1979). In subsequent *in vitro* experiments, FGF was found to be as effective as an optimal dose of newt brain extract in stimulating DNA synthesis (Mescher and Loh, 1981) and mitosis (Carlone and Foret, 1979; Carlone *et al.*, 1981). More specifically, Albert *et al.* (1987) concluded that while both acidic FGF (aFGF) and basic FGF (bFGF) behaved as mitogens for cultured blastema cells, bFGF was more effective than aFGF.

Recently, the blastema has been found to contain neurotrophic and mitogenic factors (Baudin *et al.*, 1988; Boilly, 1989). Co-culture of blastemas with spinal cord explants promoted spinal neurite outgrowth and neuronal survival; this neurotrophic effect was specific to the mesenchymal component and not the epidermal cap. On the other hand, crude blastema extracts were mitogenic for cultured blastema cells, with epidermal cap extracts being more effective than the corresponding mesenchymal extracts (Boilly and Albert, 1990). These differing blastemal effects are apparently mediated by different chemical agents (Boilly, 1989). However, aFGF may be one of the factors responsible for the blastema's mitogenic effects (Boilly, 1989; Boilly *et al.*, 1991).

Evidence suggests that an aFGF-like molecule is present in both the epidermal cap and blastema mesenchyme (Boilly, 1989; Boilly *et al.*, 1991). Furthermore, heparin, a well-documented potentiator of the mitogenic activity of aFGF, but not bFGF (Esch *et al.*, 1985), enhances the mitotic index of cultured blastema cells in a dose dependent fashion (Boilly, 1989). This result is surprising in light of the fact that heparin alone has been found to inhibit cell proliferation in a wide variety of cell types by

affecting a critical event during the mid-G1 phase of the cell cycle (Reilly *et al.*, 1989). Moreover, a polyclonal antibody against bovine aFGF stained both the epidermal cap and mesenchyme of sectioned blastemas, with mesenchymal reactivity particularly associated with the extracellular matrix. Boilly asserts that these findings argue for the presence of an endogenous aFGF-like molecule in the blastema, but maintains that this factor provides only a mitogenic and not neurotrophic activity (Boilly, 1989). Although the production site of the aFGF-like molecule is unknown, a number of potential places of origin exist: the nerves, the epidermal cap, and the mesenchymal cells of the blastema , with the extracellular matrix acting to sequester the FGF (Boilly *et al.*, 1991).

Since the extracellular matrix has been implicated in the control of cell cycle events and because FGF has been shown to induce the synthesis and secretion of various extracellular matrix components by cultured cells (Gospodarowicz, 1983), Mescher and his colleagues have examined a possible relationship between these actions. Following amputation of the amphibian limb, extracellular components of tissues are broken down, but production of hyaluronate, a large glycosaminoglycan which is a principal component of embryonic mesenchyme, begins (Mescher and Munaim, 1986). Like blastema cell proliferation, synthesis of hyaluronate is nerve dependent; in fact, it appears that these two events in regeneration are coordinately regulated, with hyaluronate production playing an integral part in the proliferative response of mesenchymal cells (Matuoka *et al.*, 1987; Mescher and Cox, 1989). Interestingly, FGF, a known blastema mitogen *in vitro*, also stimulates hyaluronate synthesis in cultured blastemas (Mescher and Munaim, 1986).

Receptor binding assays have suggested the presence of high affinity FGF receptors in the blastema mesenchyme, with low affinity binding sites in the epidermal cap; the epidermal cap also has a high level of heparan sulfate (Boilly *et al.*, 1991; Hondermarck and Boilly, 1992). Together, these

data intimate that the extracellular matrix of the epidermal cap binds and stores FGF which is then somehow released to the target high affinity receptors of the mesenchyme (Boilly *et al.*, 1991), the transduction sites of the FGF signal (Moscatelli, 1987). Other researchers have expanded this line of investigation by isolating cDNA clones representing two different members of the FGF receptor family from a newt limb blastema cDNA library: FGFR1 and both the *bek* and KGFR (keratinocyte growth factor receptor) variants of FGFR2 (Poulin *et al.*, 1991; 1993). By employing *in situ* hybridization, FGFR1 is found to be homogeneously expressed throughout the blastema mesenchyme, but is absent from the wound epithelium (Poulin *et al.*, 1993). Conversely, during the growth and blastema cell proliferation stages of the regenerate, FGFR2 is expressed in the basal layer of the wound epithelium and in the mesenchyme closely associated with the bisected bones; later, during differentiation, FGFR2 expression becomes restricted to the condensing cartilage and then the perichondrium (Poulin *et al.*, 1993). In other species it has been determined that aFGF, bFGF, and FGF-4 are ligands for FGFR1, while aFGF and either bFGF or KGF, depending on the receptor isoform, are ligands for FGFR2 (Dionne *et al.*, 1990; Johnson *et al.*, 1991; Mansukhani *et al.*, 1990;1992; Miki *et al.*,1992; Yayon *et al.*, 1992). In any case, FGF appears to function during limb regeneration and may have a mitogenic and/or angiogenic role (Boilly *et al.*, 1991).

The FGF family of growth factors also seems to play a part the developing limb bud. FGF-1 (aFGF), FGF-2 (bFGF), FGF-4, and FGF-5 each cause embryonic mouse limb outgrowth *in vitro* by stimulating proliferation of cells in distal limb mesenchyme from which the apical ectodermal ridge (AER) has been removed; other factors such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and insulin have no effect (Niswander and Martin, 1993; Niswander *et al.*, 1993). Moreover, *fgf-4*, the gene encoding the FGF-4 protein, is expressed in the

mouse AER during the period when the AER normally stimulates proliferation of underlying mesenchyme (Niswander and Martin, 1992); bFGF has been localized to the chick limb bud AER (Savage *et al.*, 1993). Recently, bFGF-loaded beads, pinned onto amputated chick wing buds, induced regeneration of digit-like structures, suggesting that the lack of regenerative ability in the chick is due to the inability of the amputated limb bud to reform a competent AER (Taylor *et al.*, 1994); this finding is particularly important in light of the fact that it represents the first time a chemically defined agent has successfully induced a regenerative response in a higher vertebrate limb. In addition, fibroblast growth factor receptors have been studied during limb bud development: FGFR1 is expressed in the mouse limb bud mesenchyme, while FGFR2 is expressed predominantly in epithelium (Peters *et al.*, 1992); these findings mimic, in part, those observed by Poulin *et al.* (1993) in the newt limb regenerate.

Perhaps the molecule fulfilling the most of Brockes (1984) requirements of a mitogen involved in nerve dependent amphibian limb regeneration is glial growth factor (GGF) (Brockes and Kintner, 1986). GGF was initially isolated from bovine brain and pituitary based on its mitogenicity for cultured Schwann cells (Brockes *et al.*, 1979; 1980). The activity, absent from non-neuronal tissue (Raff *et al.*, 1978), is most abundant in human acoustic neuromas, Schwann cell tumours associated with the auditory nerve (Brockes *et al.*, 1986). GGF-like activity has also been detected in nervous system extracts of the human, rodent, chick, frog, newt, and axolotl (Brockes, 1984; 1987b) and the newt regeneration blastema (Brockes and Kintner, 1986).

Originally reported to be a basic 31,000 Da. protein which could form a 56,000 Da. dimer (Lemke and Brockes, 1984), three major GGF isoforms have recently been purified: GGF-I (34,000 Da.), having properties similar to the previously isolated GGF, GGF-II (59,000 Da.), and GGF-III (45,000 Da.) (Goodearl *et al.*, 1993). Each of the three GGFs displays a sigmoidal

dose-dependent stimulation of Schwann cell DNA synthesis, with a half-maximal activity of 1 nM (Goodearl *et al.*, 1993); previous purifications of GGF demonstrate its mitogenicity for rat astrocytes, fibroblasts (Brockes *et al.*, 1980), and enteric glial cells (Eccleston *et al.*, 1987), but its lack of effect on oligodendrocytes and macrophage-like microglia (Brockes *et al.*, 1980). Recently, GGF-II was shown to instructively promote glial differentiation while inhibiting neuronal development of multipotent neural crest stem cells (Shah *et al.*, 1994). GGF mRNA is expressed in the embryonic spinal chord and brain (Marchionni *et al.*, 1993).

GGF-I, -II, and -III are encoded by a single gene, which appears to encode other GGF-related Schwann cell mitogens, and are generated by alternatively spliced mRNAs (Marchionni *et al.*, 1993). Moreover, other products of this same gene include the heregulin (HRG) (Holmes *et al.*, 1992) and neu differentiation factor (NDF) (Wen *et al.*, 1992), putative ligands of the c-Neu/erbB2/HER2 and erbB4/HER4 receptor tyrosine kinases (Lemke, 1993; Plowman *et al.*, 1993). The GGFs are also suggested to be neu activators (Marchionni *et al.*, 1993); interestingly, the neu (erbB2) receptor is expressed on the surface of Schwann cells where it is upregulated during Wallerian degeneration and by exposure to forskolin (Cohen *et al.*, 1992), an adenyl cyclase activator which also synergizes with the GGFs (Goodearl *et al.*, 1993). Yet another protein encoded by this gene is acetylcholine-receptor-inducing activity (ARIA), a trophic factor which stimulates the synthesis of muscle acetylcholine receptors (Falls *et al.*, 1993).

The GGFs are thought to play a role in glial cell proliferation during development and regeneration in the nervous system (Lemke and Brockes, 1984; Marchionni *et al.*, 1993). It is in the latter context that GGF has been implicated in the nerve-dependent proliferation of blastema cells in the regenerating amphibian limb. This mitogen satisfies three of the four criteria outlined by Brockes, namely: (1) GGF is present in the normally

innervated blastema, with its level increasing approximately sevenfold upon amputation and continuing to increase during the following four weeks; (2) GGF levels in the blastema decrease following denervation; and (3) both newt brain and bovine pituitary GGF increase the thymidine labelling index of 22/18 + cells in denervated blastemas *in vitro* (Brockes and Kintner, 1986). As previously mentioned, the proliferation of 22/18 + cells in the blastema is nerve dependent (Kintner and Brockes, 1985).

While most of the research in the regeneration field has focused on positive models of growth control by the nerve, Ferretti and Brockes (1991) point out that a negative growth control scenario is equally possible. Schwann cells, for example, could release a factor inhibitory to blastema cell proliferation, with production of the factor ceasing upon the return of axons to the amputation plane.

Furthermore, the means of transduction of the mitogenic signal are still to be ascertained. For example, the proliferative response of blastema cells to a neurally derived mitogen may be mediated by an elevated intracellular concentration of Ca^{2+}, which in turn causes increased cGMP levels and decreased cAMP levels; in contrast, the opposite condition, in which a calcium efflux yields elevated cAMP levels and suppressed cGMP levels is suggested to result in the depression of blastema cell divisions (Globus *et al.*, 1987). In fact, increased blastema cell cAMP levels result when the newt limb is denervated and are associated with blastema differentiation (Boilly *et al.*, 1990; 1992). Protein kinase C (PKC) is also implicated as a potential second messenger in the signal transduction of the nerve's trophic factor (Oudkhir *et al.*, 1989; Boilly *et al.*, 1990). An increase in mesenchymal PKC activity in conjunction with a translocation of this PKC activity from cytosol to membrane is correlated with blastema cell proliferation, while denervation has the opposite effect (Boilly *et al.*, 1990; 1992); similar observations were also made for PKC activity in the CNS of newts undergoing limb regeneration (Oudkhir *et al.*, 1992).

Chick Brain Growth Factor

A. Purification of CBGF

Chick brain growth factor (CBGF) was initially purified from adult chicken brain (Carlone and Rathbone, 1985). Brains were subjected to homogenization, followed by ultracentrifugation and pressure ultrafiltration through a series of Amicon Diaflo membranes of decreasing porosity (nominal molecular weight cutoffs: 30,000 Da., 2,000 Da., and 500 Da.). The biologically active material was retained on a type UM-05 membrane having a nominal molecular weight cut-off of 500 Da. This fraction was further purified by chromatofocusing. Subsequently, CBGF was purified from 18 day chick embryos by employing the same protocol of homogenization, ultracentrifugation, and pressure ultrafiltration as described for isolation of this factor from adult chick brains (Carlone *et al.*, 1987). This sequence of events was followed by DEAE (diethyl amino ethyl) ion exchange chromatography of the UM-05 retentate. Finally, the biologically active fraction isolated from the ion exchange column was submitted to reversed-phase high performance liquid chromatography (rp-HPLC) (using a shallow gradient of 0-10% acetonitrile in 0.05% trifluoroacetic acid (TFA) over a 10 minute period). Most of the active material eluted in the largest of the four UV-absorbing peaks obtained; repeated rp-HPLC of this fraction under various elution conditions continuously resulted in an apparently homogeneous single peak maintaining biological activity. Using this protocol, a purification of 25,000 fold was achieved. More recently, the UM-05 retentate was directly applied to the rpHPLC column, omitting the DEAE ion exchange chromatography step (M. Seifried, honours thesis, 1988). The separation produced at least nine UV-absorbing peaks with the most active fraction eluting in a small shoulder of a much larger peak. Interestingly, this large

peak, found by other researchers to contain an agent that stimulated chick astrocyte proliferation (Rathbone, personal communication) was determined to be inactive when tested on chick astrocytes in the aforementioned honours thesis work.

This procedure, as outlined for the purification of CBGF from embryonic and adult chick brain, has also been employed to prepare a biologically active UM-05 retentate fraction from the adult newt brain (Carlone and Rathbone, 1985). Thus, since a CBGF-like molecule also appears to be present in the nervous system of the newt, CBGF is said to lack specificity. This lack of species specificity was also characteristic of GGF (Brockes, 1984) as well as the blastema mitogens isolated by Singer *et al.* (1976) and Choo *et al.* (1978).

B. Biological activity of CBGF

CBGF was initially purified in an attempt to isolate the nerve's trophic factor involved in the control of blastema cell proliferation (Carlone and Rathbone, 1985). Earlier work had established that the trophic activity could be found in extracts of both newt peripheral nerves and brain (Lebowitz and Singer, 1970; Singer *et al.*, 1976); chick embryo brains were discovered to contain a comparable mitogenic agent which, when administered to cultured blastemas, prevented the alterations in protein synthesis characteristic of denervated blastemas *in vitro* and *in vivo* (Choo *et al.*, 1978). As a result, CBGF was first tested on cultured blastema explants and was found to be a potent stimulator of DNA synthesis with maximal activity at 10 ng/ml; the blastema tissue displayed a bell-shaped concentration dependence for CBGF activity (Carlone and Rathbone, 1985). The CBGF-like molecule partially purified as a UM-05 retentate fraction from the adult newt brain was mitogenic for newt blastema explants *in vitro* at a concentration of 100 ng/ml (Carlone

and Rathbone, 1985). In addition, CBGF has been determined to be mitogenic for the mesenchyme-like cells of cultured *Xenopus laevis* forelimb regenerates (Tsilfidis and Liversage, 1989). These cells also show a bell-shaped concentration dependence for CBGF, with the factor being most active at 1 μg/ml.

CBGF has also been found to increase the incorporation of ^{3}H-thymidine into cultured Swiss mouse 3T3 fibroblasts and astrocytes from the 10 day chick embryo brain (Carlone and Rathbone, 1985; Carlone *et al.*, 1988). Unlike the case for the blastema, 3T3 fibroblasts exhibit a sigmoidal concentration dependence for CBGF activity. CBGF also stimulated maximal division of 3T3 fibroblasts and chick embryo astrocytes when tested at a concentration of 10 ng/ml; at this concentration, a 3.5-4-fold increase in ^{3}H-thymidine incorporation over control values was realized for the chick astrocytes (Carlone *et al.*, 1988). CBGF was found to have no effect on the survival, proliferation, or differentiation of other cell types from the 10 day chick embryo brain, namely neurons and meningeal fibroblasts (Carlone *et al.*, 1988). CBGF also had no mitogenic effect on chick embryo skeletal muscle myoblasts, primary mouse embryo fibroblasts and the murine teratocarcinoma-derived STO cell line (Carlone *et al.*, 1988).

With a half-maximal stimulation (ED_{50}) of ^{3}H-thymidine incorporation into cultured chick embryo astrocytes at CBGF concentrations of approximately 5 ng/ml (or 3 nM), CBGF activity is comparable to that of a variety of other growth factors and neuropeptides (Carlone *et al.*, 1987). For example, similar values have been obtained for platelet-derived growth factor (ED_{50} = 1 ng/ml; Antoniades *et al.*, 1979) acidic fibroblast growth factor (ED_{50} approximately 40 pg/ml; Thomas *et al.*, 1984), vasopressin (ED_{50} = 1 ng/ml; Rozengurt *et al.*, 1979), and bombesin (ED_{50} = 1 nM; Rozengurt and Sinnett-Smith, 1983). This indicates that CBGF acts at physiologically relevant concentrations.

The limited array of cell types for which CBGF acts as a mitogen, parallels, in part, the small number of cell types which respond to glial growth factor. Like CBGF, GGF has been found to stimulate proliferation of astrocytes (Brockes *et al.*, 1980), fibroblasts (Brockes *et al.*, 1981), and cells of the regenerating newt limb blastema (Brockes and Kintner, 1986). However, GGF has also been ascertained to be a potent mitogen for rat Schwann cells *in vitro*, a cell type thought to be involved in the proliferative events of the blastema during Urodele limb regeneration (Raff *et al.*, 1978; Brockes, 1987a). The effect of CBGF on cultured rat Schwann cells has not been determined.

C. Biochemical characteristics of CBGF

Preliminary biochemical characteristics of CBGF have been determined. Various pieces of evidence suggest that CBGF is a basic, low molecular weight peptide (Carlone and Rathbone, 1985; Carlone *et al.*, 1987). For example, its biological activity is lost upon incubation with either trypsin (Carlone and Rathbone, 1985) or pronase (Carlone, unpublished results). Furthermore, CBGF activity is RNase and DNase insensitive (Carlone, unpublished results). Preliminary attempts to characterize the amino acid composition of CBGF, given in Table I, reveal that the factor is a polar molecule consisting of fifteen amino acids; the most predominant residues are glycine, glutamic acid/glutamine, and aspartic acid/asparagine (Carlone *et al.*, 1987). Since amino acid analysis has consistently given the same results, the possibility that this material is a random protein contaminant is negated (Carlone *et al.*, 1987). When the HPLC separation of the UM-05 retentate is monitored at 280 nm and 205 nm, the peak height of the active fraction is higher at the lower wavelength (M. Seifried, honours thesis, 1988). Since the only amino acids

Table I. Amino Acid Composition of CBGF

Amino acid	Residues/mol [a]	
	Observed	Nearest integer
Asx	2.4	2
Thr	0.4	0
Ser	1.2	1
Glx	3.7	4
Pro	0.2	0
Gly	4.3	4
Ala	0.7	1
Cys	0.8	1
Val	0.7	1
Met	0.1	0
Ile	0.1	0
Leu	0.7	1
Tyr	0.2	0
Phe	0.3	0
Trp	0.0	0
His	0.1	0
Lys	0.4	0
Arg	0.0	0
Nle	0.0	0
Total residues		15

[a] Based on an estimated molecular weight of 1500 Da.

with absorbance peaks at 280 nm are tyrosine and tryptophan, this finding supports the amino acid analysis results which indicate the absence of these two residues. Curiously, the nearest integer values for the amino acid composition indicate that no lysine or arginine residues are present, despite the fact that this mitogen is inactivated by treatment with trypsin, an enzyme cleaving peptide bonds at the carboxy side of these two amino acids (Pine *et al.*, 1980).

The purification procedure for CBGF suggests that the factor has a low molecular weight cut-off of 2,000 Da, but is retained by a membrane having a nominal molecular weight cut-off of 500 Da (Carlone and Rathbone, 1985). A comparison of the HPLC retention times and SDS polyacrylamide gel electrophoresis (PAGE) mobilities (dansylated peptides) of various standard peptides/proteins and the UM-05 retentate material indicated that CBGF had a molecular weight of 2,000-6,000 Da (Carlone and Rathbone, 1985). The amino acid composition of CBGF estimates the factor to be 1,500 Da (Carlone *et al.*, 1987), while preliminary fast atom bombardment (FAB) mass spectrometry data approximates the molecular weight at 1,600 Da (Carlone, unpublished data).

The relatively low molecular weight of CBGF distinguishes it from the blastema trophic agents isolated by Singer *et al.* (1976; less than 300,000 Da) and Choo *et al.* (1981; 13,500-47,000 Da). CBGF also appears to be smaller than some of the other well-characterized blastema mitogens such as glial growth factor (GGF-I: 34,000 Da; GGF-II: 59,000 Da; GGF-III: 45,000 Da) (Brockes, 1984; Goodearl *et al.*, 1993), acidic and basic fibroblast growth factors (each approximately 18,000 Da) (Esch *et al.*, 1985), and transferrin (76,000-81,000 Da) (Aisen and Listowsky, 1980) as well as other growth factors such as epidermal growth factor (6,045 Da) (Taylor *et al.*, 1972), and platelet-derived growth factor (30,000 Da) (Heldin and Westermark, 1989). This may suggest that CBGF is more closely related to neuropeptides such as substance P (1,500 Da), a

recognized blastema mitogen (Chang *et al.*, 1971), Hydra Head Activator (1,142 Da) (Schaller and Bodenmüller, 1981), and bombesin (1,620 Da) (Woll and Rozengurt, 1989).

CBGF activity is stable to heating at 60 °C, boiling, freezing at -70 °C, and treatment with the disulphide bond reducing agent β-mercaptoethanol (Carlone and Rathbone, 1985). The presence of only one cysteine residue, as determined by the amino acid analysis, negates the possibility of a disulphide linkage in CBGF (Carlone *et al.*, 1987). Although CBGF is stored dry and frozen at -70 °C, it loses activity upon repeated freezing and thawing (R. Carlone, personal communication).

D. Possible nucleotide content of CBGF

When a sample of CBGF was obtained from M. Rathbone and his colleagues for further identification, it was found not to contain a peptide, but be primarily composed of guanosine 5'-monophosphate (GMP), as determined by ^{1}H- and ^{31}P- nuclear magnetic resonance (NMR) spectroscopy (M. Seifried, honours thesis, 1988). This sample had no biological activity when tested using the astrocyte biosassay.

Preliminary 1H-NMR analysis indicated the presence of a nucleoside/nucleotide component in those rpHPLC fractions found to be most ineffective in the astrocyte bioassay; such components appeared to be absent from mitogenic fractions (M. Seifried, honours thesis, 1988).

Rathbone and his colleagues later concluded that the most mitogenic of the components which they isolated from the 18-day chick embryo brain and purified by rpHPLC was GMP with a molecular weight of 363 Da. (Kim *et al.*, 1991); this conclusion was based upon a comparison of rpHPLC retention times with ultraviolet absorption spectra, thin layer chromatography plates, ^{1}H- and ^{31}P-NMR spectra, and FAB mass spectra for both the chick brain-isolated fraction and commercial GMP. They also

found that both the chick brain-isolated material and commercial GMP were mitogenic for 10-day chick embryo astrocytes *in vitro* ; both substances stimulated ^{3}H-thymidine incorporation into these cells in a dose-dependent fashion, with maximal activity at 50 µm (Kim *et al.*, 1991).

Similarly, these researchers have isolated adenosine 5'-monophosphate (AMP; 347 Da.) from the 18 day chick embryo brain and verified its identity with commercial AMP using the same methods employed in their GMP study; AMP was also found to be a mitogen for 10 day chick embryo brain astrocytes, stimulating maximum ^{3}H-thymidine incorporation into these cells at 500 nM (Rathbone *et al.*, 1992a).

Moreover, these researchers found that guanosine, guanosine diphosphate (GDP), guanosine triphosphate (GTP) (Kim *et al.*, 1991), adenine, adenosine, adenosine diphosphate (ADP), adenosine triphosphate (ATP) (Rathbone *et al.*, 1992a), deoxyguanosine, dGMP, dGDP, and dGTP, deoxyadenosine, dAMP, dADP, and dATP, as well as some adenosine metabolites such as inosine, hypoxanthine, xanthosine 5'-monophosphate (XMP), XDP, and XTP (Christjanson *et al.*, 1993) were also mitogenic for the astrocytes; while these purines were found to be astrocyte mitogens, pyrimidine nucleosides, nucleotides (with the possible exception of uridine diphosphate), and deoxynucleotides were inactive (Christjanson *et al.*, 1993). Guanosine, adenosine, and their nucleotides also stimulate proliferation of 10 day chick embryo brain meningeal fibroblasts, human brain capillary endothelial cells, Swiss mouse 3T3 fibroblasts, and the human astrocytoma cell lines SKMG-1 and U373, in addition to the 10 day chick embryo brain astrocytes (Rathbone *et al.*, 1992c); as previously stated, CBGF has no effect on these meningeal fibroblasts, indicating that this factor is not GMP or AMP (Carlone *et al.*, 1988). Generally, guanosine and its analogues were more active in stimulating cell proliferation than the adenosine-based compounds (Rathbone *et al.*, 1991; 1992c).

The use of various purinergic receptor antagonists has suggested that

adenosine and guanosine mediate their mitogenic effect via activation of an adenosine A_2 receptor, while their nucleotides employ purinergic P_{2y} receptors (Rathbone *et al.*, 1992a,c); these results further imply that guanosine, adenosine, and their nucleotides are not merely behaving as nutritional supplements. Since guanosine is not known to bind to purinergic receptors, these authors surmise that it works indirectly by inhibiting the action of adenosine deaminase on extracellular adenosine (Rathbone *et al.*, 1992c,e); adenosine is readily released from hypoxic, damaged, or dying cells (Stone *et al.*, 1990; Rathbone *et al.*, 1992d).

Using a different purification method than that employed to isolate GMP and AMP from the 18 day chick embryo brain, Rathbone and his colleagues (1992b) separated eight partially purified fractions mitogenic for cultured chick astrocytes: 24, 17, 12, 9, 5, 2.8, 1.4, and 1.2 kD. One of the lower molecular weight fractions may represent CBGF.

The candidates for the nerve-elaborated blastema mitogen involved in newt limb regeneration which have been discussed include substance P (Globus *et al.*, 1991), hydra head activator (Fuentes *et al.*, 1993), transferrin (Mescher and Kiffmeyer, 1992), acidic and basic fibroblast growth factors (Boilly *et al.*, 1991; Albert *et al.*, 1987), glial growth factor (Brockes and Kintner, 1986), and chick brain growth factor (Carlone *et al.*, 1987). Of these, the best possibilities for such a trophic factor are represented by transferrin, aFGF, bFGF, GGF, and CBGF. However, although transferrin, aFGF, and bFGF are well-characterized molecules available in sufficient quantities, little research using these factors has been directed specifically at addressing the questions posed by Brockes (1984) in his four criteria required of a blastema mitogen. The originally isolated GGF was found to meet three of these requirements, but the three GGF isoforms (GGF-I, GGF-II, and GGF-III) (Goodearl *et al.*, 1993) subsequently purified have yet to be tested. In an effort to eliminate or substantiate claims to a

role in the nerve-dependent blastema cell proliferation phase of limb regeneration, experiments aimed at testing whether these factors meet Brockes' four criteria would be extremely valuable. In addition, once mitogens such as CBGF are more abundantly available and more fully characterized, experiments of this nature may also be carried out in an effort to determine this factor's potential part in Urodele limb regeneration.

METHODS AND MATERIALS

Purification of Chick Brain Growth Factor (CBGF)

A. Initial isolation and ultrafiltration

The purification protocol of CBGF has been previously described (Carlone *et al.*, 1987); the following procedure represents an improvement of that method.

Ten or twenty dozen live eighteen-day chick embryos were decapitated and the brains were removed and homogenized 1:1 (w:v) in cold 0.02 M potassium phosphate buffer (pH 7.3) using a 50 ml glass homogenizer. The homogenate was then centrifuged at 12,500 g for 20 minutes at 4°C. The supernatant was filtered through glass wool and then ultracentrifuged at 105,000 g for 1.5 hours at 4°C. Supernatants were again filtered through glass wool and then passed through a series of Amicon Diaflo membranes of decreasing porosity. This ultrafiltration was accomplished at 4°C using a pressure of approximately 35-40 psi. The filtrate from the first membrane, PM-30 (nominal molecular weight cut-off of 30,000 Da.) was applied to the second membrane, YM-2 (nominal molecular weight cut-off of 2,000 Da.). The YM-2 filtrate was lyophilized and stored at -70°C prior to further purification of the active component.

B. Size exclusion chromatography

The YM-2 filtrate was further fractionated by eluting it from a size exclusion column. Biogel P6 (nominal molecular weight cut-off of 6,000 Da.) column packing was equilibrated against 0.05 M ammonium acetate. This was used to prepare a 36x1.1 cm column with an approximately 35 ml bed volume. The dry YM-2 filtrate was reconstituted

in approximately 1.5 ml of the ammonium acetate buffer and filtered through a 0.2 μM filter. The sample was eluted at a flow rate of approximately 0.165 ml/min with 10 minute fractions being collected. The ultraviolet (UV) absorbance of the fractions was monitored at 260 nm and 280 nm and the peaks were pooled, lyophilized, and stored at -70°C.

C. Anion exchange high performance liquid chromatography (HPLC)

Anion exchange chromatography was achieved using a Waters anion exchange column. The active fraction, P6-3, from the Biogel P6 column was reconstituted in distilled H_2O and fractionated with the following gradient: 0.1% 0.007 M KH_2PO_4 and 0.007 M KCl (pH 4.0) to 99.9% 0.25 M KH_2PO_4 and 0.50 M KCl (pH 5.0) in 20 minutes. The solvents were filtered and degassed. A flow rate of 1.5 ml/min and a chart speed of 0.5 cm/min were employed. The elution profile was monitored primarily at 256 nm, but also at 280 nm and 229 nm. Fractions were collected manually, pooled, lyophilized, and stored at -70°C. Following the anion exchange HPLC elutions performed on any given day, the system was thoroughly flushed with distilled H_2O in order to remove all buffer salts and prevent corrosion of the HPLC hardware. The column was stored in CH_3CN.

D. Reverse phase high performance liquid chromatography (rp-HPLC)

Reverse phase HPLC was used to further purify the mitogen from the P6-3 fraction of the Biogel P6 column elution. The sample was dissolved in distilled H_2O and a Waters μ Bondapak C_{18} column was used in the separation. Fractionation was accomplished with a fairly steep gradient of 0.1% to 99.9% acetonitrile (CH_3CN) in a doubly glass distilled H_2O with

0.05% trifluoroacetic acid (TFA) in 20 minutes; a separation was also achieved with an initial 2 minute period in which distilled H_2O with 0.05% TFA was run isocratically prior to the onset of the gradient. The solvents were filtered and degassed. A flow rate of 1.5 ml/min and a chart speed of 0.5 cm/min were used. Pure CH_3CN was employed to purge and store the column. To prevent precipitation of the TFA, the column was purged with a 50:50 mixture of CH_3CN:H_2O and then distilled H_2O alone before the initial elution on any given day; after the final elution of the day, this regime was followed in reverse and the column was stored in CH_3CN. The elution profile was monitored primarily at 280 nm, but also at 256 nm. Peaks were collected manually, pooled, lyophilized, and stored at -70°C.

Both guanosine 5'-monophosphate (5'-GMP) and adenosine 5'-monophosphate (5'-AMP) were eluted under the conditions employed for the fractionation of fraction P6-3.

Cell Culture

A. Isolation of non-neuronal cells - primary cultures

The non-neuronal cells used in the bioassays to determine the biological activity of CBGF were isolated by the method of Hanson *et al.* (1982). Approximately six 10 day chick embryos were decapitated and the cerebral hemispheres were dissected into Ca^{2+} and Mg^{2+} free phosphate buffered saline (PBS). The meninges were removed and discarded. After mincing, the tissue was trypsinized with 5 ml 0.25% trypsin (which had been diluted with PBS from a 10X stock solution) for 30 minutes at 37°C. The trypsin was then neutralized with 5 ml 10% fetal bovine serum (FBS) in complete alpha-minimal essential medium (α-MEM) from GIBCO (described later). The suspension was centrifuged at 1000 rpm for 5 minutes, the supernatant was removed, and the pellet was

resuspended in fresh medium. The tissue was further dissociated by trituration (10X) with a Pasteur pipette followed by mechanical sieving through a 63 μm Nitex mesh. The cell suspension was equally divided among four to six 60 mm collagen-coated Falcon culture dishes with each dish containing a total of 5 ml of complete medium with 10% FBS. Cells were incubated at 37°C and 2% to 5% CO_2. The non-neuronal cells were selected by differential adhesion to the collagen-coated culture dishes. This was accomplished by agitation of the plates for approximately 2 minutes at 30 minute intervals for 4 hours. At this time, the non-neuronal cells were affixed to the collagen substrate and the neuronal cells had formed aggregates in the medium. The medium was gently aspirated, the attached cells were washed with PBS, and 5 ml fresh complete medium with 10% FBS was added. Any remaining neuronal cells were lost upon subsequent subculturing. The non-neuronal cells were maintained in α-MEM supplemented with 33.3 mM D-glucose, 1x antibiotic/antimycotic (GIBCO), 5 μg/ml insulin, 5 μg/ml human transferrin, $2x10^{-8}$ M progesterone, 100 mM putrescine, $3x10^{-8}$ M sodium selenate (complete medium) and 10% FBS. The non-neuronal cells isolated and prepared in this way were referred to as primary cultures.

B. Collagen-coating of culture dishes

A 0.5 mg/ml collagen solution was prepared by dissolving bovine achilles tendon collagen (GIBCO) in a 1:1000 mixture of glacial acetic acid:distilled H_2O. After stirring for 24 hours, the solution was centrifuged at 1000 rpm for 5 minutes and the pellet was discarded. Culture dishes were coated with a small amount of collagen for one hour at which time the excess collagen was removed and the plates were allowed to dry. Before cells were plated onto the dishes, the dishes were washed with PBS.

C. Subculturing of non-neuronal cells

When the primary cultures of non-neuronal cells had reached confluence, usually after two to four days, the cells were passaged. The medium was removed from the primary culture dishes, they were washed with PBS, and trypsinized with 1 ml 0.25% trypsin for 8 minutes at 37°C. The trypsin was prepared as previously described. At this point, 5 ml complete medium with 10% FBS was added to the dishes to neutralize the trypsin. By trituration, the cells were lifted from the plates and the resulting cell suspension was centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded and the pellet was resuspended in complete medium containing 10% FBS by trituration (10X) with a Pasteur pipette. Large pieces of the cell sheet that were resistant to trituration were permitted to settle by gravity. The supernatant was divided equally among 60 mm Falcon culture dishes. The cells were incubated at 37°C and 2% to 5% CO_2. Cells were confluent after approximately two to three days at which time they were subcultured again.

Growth Factor Bioassay

A. General bioassay

Non-neuronal cells used for CBGF and nucleotide bioassays were passaged and seeded into collagen-coated 24 well plates (Falcon) at a density of $3x10^4$ cells/well. Experiments determining the optimum bioassay conditions employed cells seeded at a density of $1.2x10^5$ cells/well into collagen-coated 35 mm Falcon culture dishes. Immunohistochemical studies to determine the number of GFAP+ cells in the culture employed cells seeded at a density of $1.2x10^5$ cells/dish into 35 mm culture dishes containing collagen-coated coverslips. Cells were

grown to confluence in the centre of the wells using complete medium with 10% FBS. At this time, the cells were rendered "quiescent" with complete medium containing either 0.1% or 1% FBS. After the 24 hour "quiescence" period, compete medium with 0.1% or 1% FBS containing varying concentrations of the test factor replaced the medium in the wells. Several replicates of each concentration were used in the assay. The concentrations of CBGF samples to be tested were estimated based on their protein content as determined by the Lowry assay. In addition, several wells with complete medium containing 0.1% or 1% FBS and others with 10% FBS were included as controls in each bioassay. The non-neuronal cells were stimulated for 6 hours before 1 μCi/well of methyl-^{3}H-thymidine (specific activity 50 Ci/mmole; ICN) was added for a further 24 hr stimulation/labeling period. The medium was then aspirated, the cells were washed 2X with cold PBS, and the cells were fixed in 10% trichloroacetic acid (TCA) for 10 minutes at 4°C. The cells were washed with cold 5% TCA and then 2X with cold PBS; the cells were then air-dried. The dried cells were solubilized in freshly prepared 0.5% SDS in 0.3N NaOH. The TCA precipitable material was precipitated with 25% TCA/well for at least 20 minutes at 4°C. The well contents were filtered through glass fibre filters (Whatman GF/C), washed with cold 5% TCA (2X 1 ml, 1X 8 ml), and finally rinsed with ethanol. Filters were dried and counted in 7 ml Aqueous Counting Solution (ACS) in a Beckman scintillation counter (model LS 1800).

B. Pronase sensitivity tests

Pronase sensitivity tests were carried out on fraction P6-3. A Lowry protein estimation was performed and a quantity of P6-3 resulting in a final test concentration of 30 ng/ml was used. This was incubated with pronase at a P6-3:pronase concentration of 100:1 for 10 minutes at 37°C.

A control employing distilled H_2O in place of the P6-3 was also prepared. These samples were then diluted in complete medium with 1% FBS to a final concentration of 30 ng/ml P6-3 and added to the "quiescent" cells. The remainder of the bioassay was carried out as described previously.

C. Calculation and presentation of bioassay data of tested fractions

The biological activity of test fractions was determined using the bioassay as described. Fractions were tested in twenty-four well plates with each well representing one sample. Each test plate included two controls, 1% FBS (occasionally 0.1% FBS) and 10% FBS. Test fractions were added to medium supplemented with 1% FBS. Incorporated ^{3}H-thymidine was measured as counts per minute (cpm) on a scintillation counter. Several replicates of each fraction were tested and the cpm values of these were averaged. To facilitate a comparison of the effect of a given fraction on cell growth with 1% FBS, data were calculated as percent difference from the 1% FBS control using the following formula:

$$\% \text{ difference from 1\% FBS} = \frac{\bar{X} \text{ cpm cells with test fraction} - \bar{X} \text{ cpm cells in 1\% FBS}}{\bar{X} \text{ cpm cells in 1\% FBS}} \times 100\%$$

where $\bar{X}$ represents the mean of three to six replicates of a specific treatment in the same assay. The biological activity of the test fractions is presented in bar graph form with each treatment listed on the horizontal axis and its effect on cell growth represented as percentage difference from the 1% FBS control on the vertical axis. Thus, 1% FBS is depicted as the horizontal axis of the graph, that is, 0% difference from the control. Each graph also includes the effect of 10% FBS, a known growth stimulus. To ascertain whether a statistically significant difference ($p \leq 0.05$) existed

between the proliferative effects of any one test treatment and 1% FBS, the Wilcoxon Mann-Whitney U test was applied. Since the data was not normally distributed, a non-parametric unpaired test was deemed most suitable. A statistically significant result is graphically presented as an "x" over the corresponding bar on the graph. All bioassay results are presented in this manner unless otherwise specified.

Glial Fibrillary Acidic Protein Immunohistochemistry

Using the indirect immunofluorescence method, the non-neuronal cells were tested for their expression of glial fibrillary acidic protein (GFAP). Cells, seeded at a density of $1.2x10^5$ cells/dish, were cultured on collagen-coated coverslips in 35 mm Falcon petri dishes and were used in these studies while still subconfluent. The cells were washed with Hank's buffered saline and then fixed in 4% paraformaldehyde-0.25% gluteraldehyde in 0.1M cacodylate buffer for 10 minutes at 4°C. The cells were again washed in Hank's buffered saline, the coverslips were removed, and the cells on the coverslips were incubated with acetone for 1-2 minutes at -20°C. The washed cells were incubated with 10% FBS for 45 minutes at room temperature and washed again in Hank's buffered saline. The cells were then incubated with a 1:500 dilution of mouse monoclonal antibody (IgG) to pig GFAP (ICN) (1° antibody) for two hours at room temperature; the stock anti-GFAP had been diluted with 0.1% bovine serum albumin (BSA)-0.1% sodium azide in Hank's buffered saline. Control cells were incubated with the appropriate dilution of BSA in Hank's buffered saline (no 1° antibody). After this period of time, the coverslips were dipped in Hank's buffered saline and then washed 3X for 3 minutes with shaking. The coverslips were then incubated with a 1:100 dilution of rabbit antibody to mouse IgG (2° antibody) for one hour at 37°C. The cells were washed using the method employed in the wash following the

incubation with the 1° antibody. The coverslips were then incubated with a 1:100 dilution of rhodamine-labelled goat antibody to rabbit IgG (3° antibody) for 1 hour. A control coverslip which had been treated with both the 1° and 2° antibodies was incubated with Hank's buffered saline alone (no 3° antibody). The cells were again washed using the method employed following the 1° antibody incubation. The coverslips were mounted onto glass slides using 50% glycerol, 50% PBS with 1% (w:v) DABCO. Using a Zeiss photomicroscope III equipped for rhodamine epifluorescence, the coverslips were examined for GFAP-positive cells. Both of the controls, in which the 1° antibody and 3° antibody were separately omitted, revealed no detectable fluorescence.

Nuclear Magnetic Resonance Spectroscopy

The sample rp-HPLC fraction #7 was dissolved in 99.96% D_2O and placed in a micro NMR tube. A Bruker AC 200 instrument was used to perform the ^1H NMR studies. The HDO resonance line at 4.6 ppm was used as a reference.

Fast Atom Bombardment Mass Spectrometry

FAB-MS were obtained with a KRATOS concept 1S double-focusing mass spectrometer. For normal FAB-MS, samples were dissolved in 99.5+% glycerol (spectrophotometric grade) and transferred to the probe tip. The probe tip used for nucleotide work was 1.5-2 mm in diameter; a mega probe five times this size was employed in acquiring the spectra of bombesin and rp-HPLC fraction #7. The probe was then inserted into the FAB-MS ion source. For flow FAB-MS, the samples were dissolved in 5% glycerol in H_2O (v:v). Sample introduction occurred through a 0.075 mm fused silica tubing which erupted 0.25 mm from the centre of the probe tip

surface. The sample flow rate was 5-10 µl/min at the probe tip. Under both normal and flow FAB-MS systems, the following conditions were employed: stainless steel targets at 8 kev acceleration potential; xenon bombardment gas with a xenon particle energy of 7.5 Watts; spectral range, m/z 100 to m/z 2000; resolving power, 1500. Mass calibration was done using both perfluoroheptatriazine (about 1500 Da) and cesium iodide as mass references.

RESULTS

Optimization of Bioassay Conditions

The bioassay employed in this work was used to determine the biological activity of CBGF at various levels of purity and of several nucleotides. This involved culturing non-neuronal 10 day chick embryo brain cells, rendering them "quiescent", adding the factor to be tested, and later adding ^{3}H-thymidine. Cell proliferation in response to the exogenous factor was indirectly measured using a scintillation counter to determine radioactive label uptake into the cells' DNA.

The first goal of this research was to partially characterize and optimize the culture and assay conditions of this system. The systematic study of these parameters had not been done previously.

Culture Conditions

A. Collagen-coated versus uncoated culture dishes

Initially, non-neuronal cell growth on collagen-coated and uncoated culture dishes was qualitatively examined.

Cells plated onto collagen-coated culture dishes readily adhered and flattened. This was not the case for the cells placed in uncoated dishes. At confluence of primary cultures on collagen-coated plates, cells plated at an equal density on uncoated plates were extremely sparse. After the first sub-culture of cells which had been grown on collagen-coated dishes, those cells plated on uncoated dishes took longer to adhere and flatten. Despite growing faster as first passage cells than as primary cultures, non-

neuronal cells on uncoated dishes were consistently more sparse than their counterparts plated onto collagen-coated dishes.

B. Responsiveness of different subcultures of non-neuronal cells to 10% FBS

Throughout the course of this research, each bioassay performed included two controls: one in which cells were at a low basal level of growth in medium supplemented with 0.1% or 1% fetal bovine serum (FBS) and the second in which cells were at nearly maximal growth in medium supplemented with 10% FBS. The percent difference of ^{3}H-thymidine incorporation in cells treated with 10% FBS with respect to those treated with the basal level of FBS was calculated as:

$$\% \text{ Difference from low FBS control} = \frac{\overline{X} \text{ cpm cells in 10\% FBS} - \overline{X} \text{ cpm cells in low FBS}}{\overline{X} \text{ cpm cells in low FBS}} \times 100\%$$

where $\overline{X}$ represents the mean of three to six replicates of a given treatment in the same assay and cpm represents counts per minute as determined by the ^{3}H channel of a Beckman scintillation counter.

The percent difference of 10% FBS from the low FBS control was used as a measure of the cells' ability to respond to a known proliferative growth stimulus (ie. 10% FBS). Data from assays employing the 1% FBS control were used to compare the responsiveness of the non-neuronal cells at each subculture within every cell preparation. Employing Wilcoxon one-tailed and two-tailed paired-sample tests (Zar, 1984), it was found that first subculture cells were less responsive to stimulation than second subculture cells derived from the same cell isolation ($0.02 < p < 0.05$). No difference in the ability to stimulate second subculture and third subculture cells was detected using this statistical test ($p=0.1$). Several

assays in which fourth subculture cells were tested showed no statistical difference (using the Wilcoxon Mann-Whitney U test) between the 1% and 10% FBS controls. The results of such assays were discounted. Thus, a comparison of third and fourth subculture cells by the Wilcoxon paired sample test could not be made since the remaining sample size was insufficient.

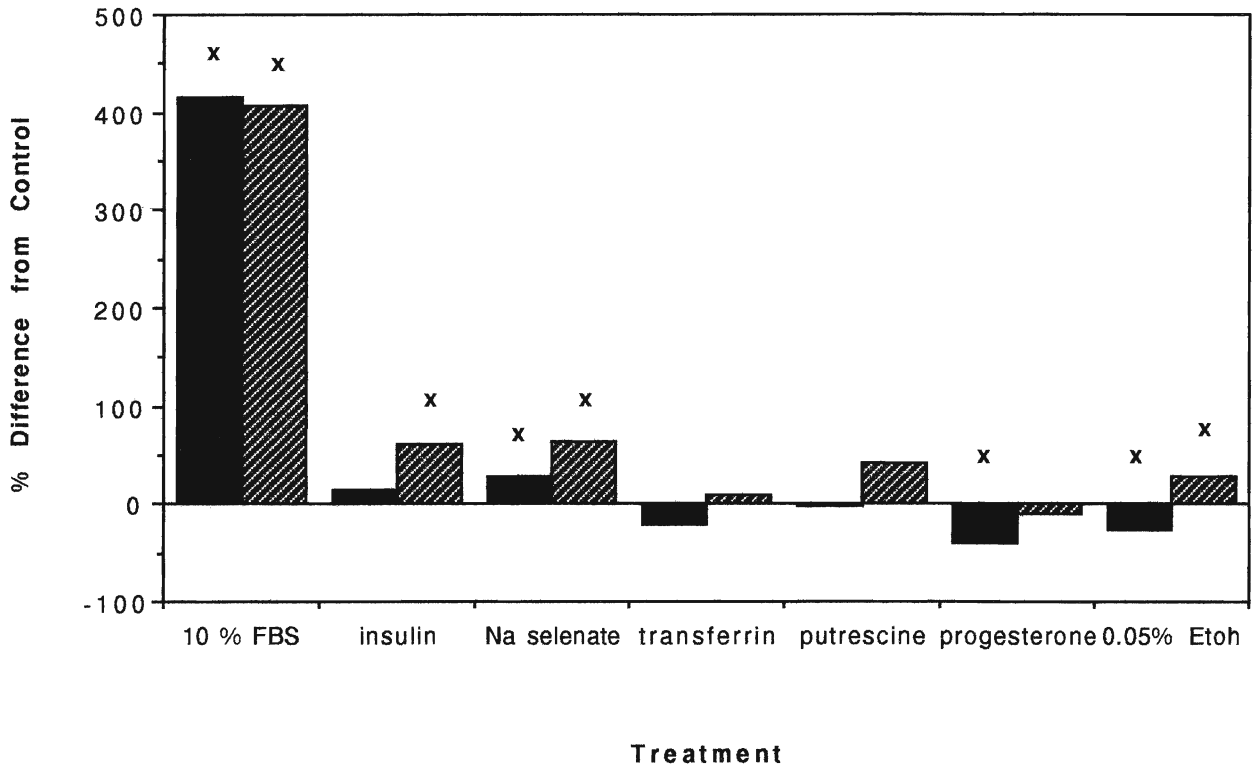
C. Effects of medium components on non-neuronal cell growth

The maintenance of non-neuronal cell cultures in this laboratory had relied upon the use of a supplemented undefined medium consisting of an alpha minimal essential medium (α-MEM) base with fetal bovine serum (FBS) , 33.3 mM D-glucose, 1 x antibiotic/antimycotic, 5 μg/ml insulin, 3×10^{-8} M sodium selenate, 5 μg/ml transferrin, 100 μM putrescine, and 2×10^{-8} M progesterone (Carlone *et al.*, 1987). The individual effects of some of these additives on ^{3}H-thymidine incorporation in these cells was investigated.

Non-neuronal cells were grown in 10% FBS in α-MEM supplemented with glucose and antibiotic/antimycotic. They were then rendered quiescent in the same medium with 0.1% FBS rather than 10% FBS. Insulin, sodium selenate, transferrin, putrescine, and progesterone were individually added to 0.1% FBS α-MEM and separately tested on the cells. Since the progesterone had been dissolved in ethanol, a control of 0.05% ethanol was included in the assay. The assay was performed twice, each with three replicates of the test substances. (In plate B, only two replicates of the 0.05% ethanol treated cells were used). The results are presented in Figure 1. A Wilcoxon Mann-Whitney U test (Zar, 1984) was applied to the data to find that, in both plates, 10% FBS and sodium selenate significantly stimulated ^{3}H-thymidine incorporation in the cells. For 10% FBS, this increase was 406.6% to 414.2% and for sodium selenate

Figure 1. Effects of Various Medium Components on 3H-thymidine Incorporation in Two 24-well Plates of Cultured Chick Embryo Brain Non-neuronal Cells.

Data are presented as percent difference from the 0.1% FBS control for each treatment on each of plates A (solid bars) and B (hatched bars). Statistically significant differences ($p \leq 0.05$) are represented by an "x" over the corresponding bar on the graph. Etoh denotes ethanol, used to dissolve the progesterone and as an assay control. n = 3 for each test substance except plate B Etoh where n = 2. (FBS = fetal bovine serum)



0% represents 0.1% FBS control

it was 28.0% to 63.7% over the 0.1% FBS control. In plate B, insulin was found to affect a 60.1% increase in radiolabel uptake. Progesterone was found to be inhibitory (-41.9%) from the results of plate B. Conflicting responses were noted for the addition of 0.05% ethanol which was inhibitory (-27.5%) to growth in the cells of plate A, but stimulatory (26.3%) to the cells of plate B. All other medium additives had no statistically significant effect on the cells.

A comparison of incomplete medium containing only the α-MEM, FBS, glucose, and antibiotic/antimycotic and complete medium containing the aforementioned ingredients as well as each of the additives insulin, sodium selenate, transferrin, putrescine and progesterone was made. The percent difference of 10% FBS from 0.1% FBS was utilized to contrast five replicates of complete and six replicates of incomplete medium. One-tailed and two-tailed Mann-Whitney U tests indicated that the complete medium significantly stimulated ^{3}H-thymidine incorporation above that observed for cells treated with incomplete medium ($p \leq 0.05$).

D. Response of non-neuronal cells to 0.1% and 1% FBS

At the outset of this research, medium supplemented with 0.1% FBS was used to render the non-neuronal cells "quiescent" and to serve as the base to which cell test factors were added. A low percentage of FBS was expected to keep the cells viable and growing at a slow rate, while permitting the difference between the cells' response to 0.1% FBS and 10% FBS to be maximized. Each assay included a control in which only 0.1% FBS, without an additional test factor, was added to the cells. Incorporation of ^{3}H-thymidine in these controls was very low. Moreover, in a bioassay in which cells were tested with 0%, 0.1%, 1%, and 10% FBS, cells in the presence of 0% and 0.1% FBS appeared less healthy, with greater cell death occurring, than cells treated with either 1% or 10% FBS.

The percent difference in ^{3}H-thymidine incorporation in cells tested with 10% FBS over those in 0.1% FBS was 515.3%, while the percent difference of cells in 10% FBS over those in 1% FBS was 393.0%. Although one of the goals of quiescence is the maximization of the difference between cell growth in the "quiescent" state and in 10% FBS, use of 1% FBS instead of 0.1% FBS, for this purpose was deemed more suitable in light of its positive effect on non-neuronal cell health.

Assay Conditions

A. The effect of varying the duration of "quiescence"

Non-neuronal cells were grown in complete medium supplemented with 10% FBS; this allowed the cells to proliferate. Before the cells could respond to stimulation by a test factor, it was necessary to decrease their rate of growth, that is to say, to render them "quiescent". True quiescence is a state in which the cells remain viable but do not divide. The non-neuronal cells in this laboratory were found to be visibly "healthier" in a medium supplemented with FBS. Therefore, when the cell culture medium was changed from 10% FBS to 0.1% or 1% FBS, the cells were not rendered truly quiescent, but were growing at a basal level. This step in the bioassay permitted the difference between the cells' response to 10% FBS and 0.1% or 1% FBS to be maximized. A test substance could then be added to 0.1% or 1% FBS to determine the cells' reaction to it.

The optimum length of "quiescence" was studied using 1% FBS. Individual 35 mm culture dishes had varying quiescence durations from three to seventy-two hours (Figure 2), with each time having four replicates. Incorporation of the ^{3}H-thymidine was highest at three hours, decreased by six hours, and was at a constant level by 14.5 hours. This

Figure 2. The Effect of the Duration of "Quiescence" Time on 3H-thymidine Incorporation in Cultured Chick Embryo Brain Non-neuronal Cells.

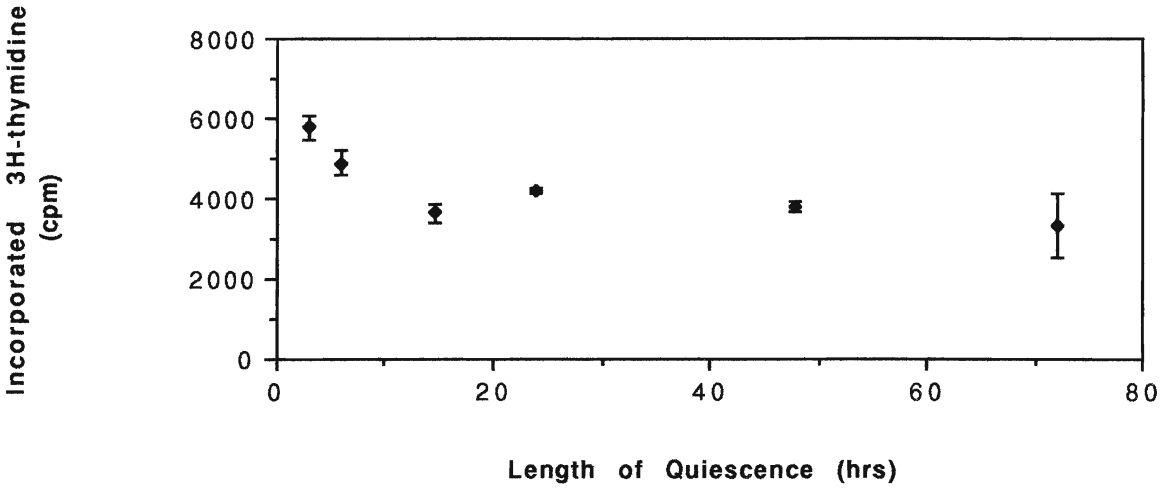
Data are presented as means ± standard deviation of the incorporated ^{3}H-thymidine in cpm for each time tested, where n = 4. Cells were rendered "quiescent" in 1% FBS for varying lengths of time, followed by a constant 24 hr. prestimulation time in 10% FBS and a subsequent constant 24 hr. labelling time. (cpm = counts per minute; FBS = fetal bovine serum)

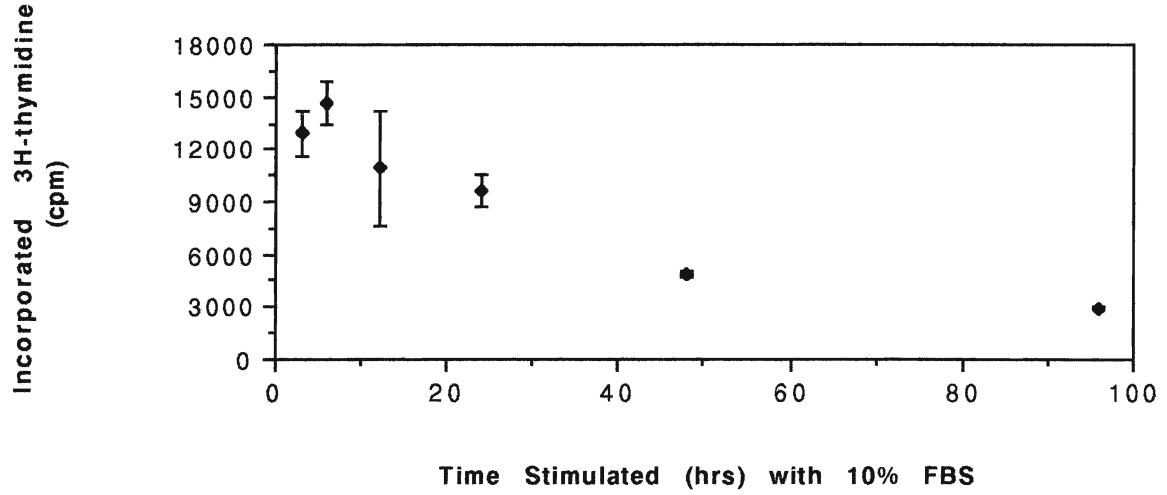
Figure 3. The Effect of the Duration of "Prestimulation" Time on 3H-thymidine Incorporation in Cultured Chick Embryo Brain Non-neuronal Cells.

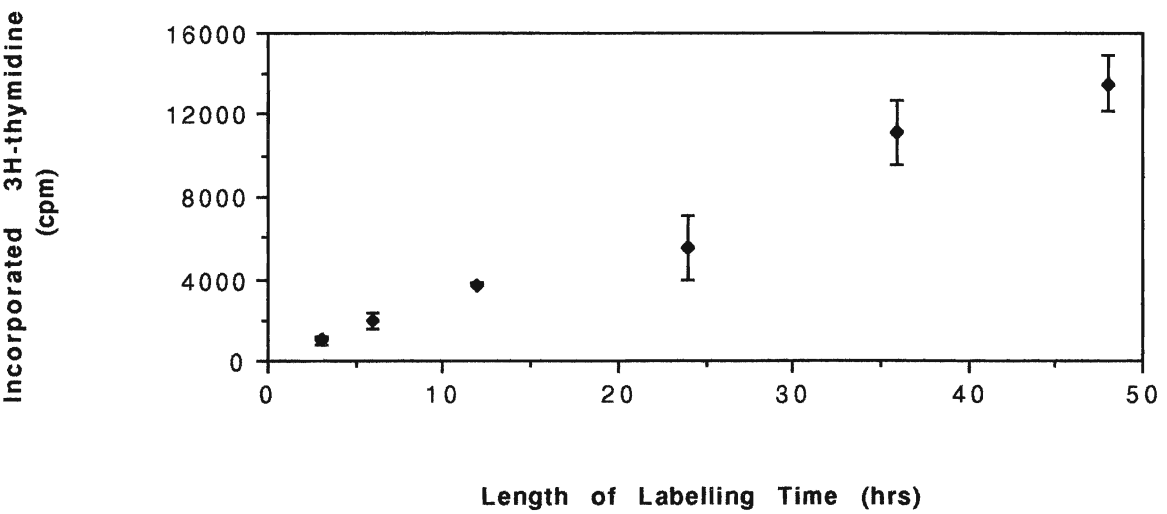
Data are presented as means ± standard deviation of the incorporated ^{3}H-thymidine in cpm for each time tested, where n = 4. Cells were rendered "quiescent" in 1% FBS for a constant 24 hr. period, followed by a prestimulation time of varying lengths in 10% FBS and a subsequent constant 24 hr. labelling time. (cpm = counts per minute; FBS = fetal bovine serum)

Figure 4. The Effect of the Duration of 3H-thymidine Labelling Time on 3H-thymidine Incorporation in Cultured Chick Embryo Brain Non-neuronal Cells.

Data are presented as means ± standard deviation of the incorporated ^{3}H-thymidine in cpm for each time tested, where n = 4. Cells were rendered "quiescent" in 1% FBS for a constant 24 hr. period, followed by a constant 24 hr. prestimulation time in 10% FBS and a subsequent labelling time of varying length. (cpm = counts per minute; FBS = fetal bovine serum)


Incorporated 3H-thymidine (cpm)
8000
6000
4000
2000
0
0
20
40
60
80
Length of Quiescence (hrs)


Incorporated 3H-thymidine (cpm)
18000
15000
12000
9000
6000
3000
0
0
20
40
60
80
100
Time Stimulated (hrs) with 10% FBS


Incorporated 3H-thymidine (cpm)
16000
12000
8000
4000
0
0
10
20
30
40
50
Length of Labelling Time (hrs)

constant level of cell growth was maintained until the last test time of seventy-two hours "quiescence". It appeared that at three and six hours of quiescence, the cells were continuing to respond to residual effects of having been in 10% FBS. By 14.5 hours, the difference between growth in 1% and 10% FBS had reached a maximum which was maintained until at least seventy-two hours. Twenty-four hours "quiescence" fell in this constant range and was employed in future assays as a convenient length of time. For the "quiescence" time course study, constant 24 hour stimulation and labelling times were used.

B. The effect of varying stimulation time

Following the time of "quiescence", the non-neuronal cells of this bioassay were at a low level of growth and ready to respond to a growth stimulus. It was at this point in time that a known concentration of a test substance was mixed with 0.1% or 1% FBS and added to the cells so that its effect on their proliferation might be determined. Since the cells were initially grown in medium supplemented with 10% FBS, this was known to be an effective growth stimulus with which to study the optimum length of stimulation.

Individual 35 mm culture dishes were each rendered "quiescent" in 1% FBS for a constant time of 24 hours. After this, 10% FBS was added to the dishes, however the duration of "prestimulation", without added radioactive label, was varied from three to ninety-six hours (Figure 3), with each time represented with four replicates. A constant labelling time of 24 hours ensued. Incorporation of ^{3}H-thymidine increased to a high at six hours of prestimulation. This was followed by an initial rapid decrease in label incorporation to twelve hours and then a lower rate of decline to ninety-six hours of prestimulation. It appeared that the non-neuronal cells of this study required approximately six hours of contact with 10%

FBS without added ^{3}H-thymidine before the cells could achieve a maximum stimulation response. Since there is a decline in growth after this time, it is possible that the proliferating cells are depleting the medium of the stimulus. As a result, a six hour period of prestimulation was employed in future assays. It should be noted that this optimal stimulation time may apply specifically to treatment with 10% FBS and could conceivably differ for treatment with other substances such as those used in this research.

C. The effect of varying labelling time

In order for the degree of growth caused by a given test factor to be determined, it was necessary to have a method of detecting newly formed cells. For this reason, a radioactive label that would incorporate into proliferating cells was required. ^{3}H-thymidine was added to the medium after the initial stimulation period. By adding a labelled nucleoside to the non-radioactive nucleoside pool, proliferating cells undergoing DNA replication incorporated these components into their DNA and were marked. The extent of ^{3}H-thymidine incorporation, and thus DNA synthesis, was then determined as counts per minute in a scintillation counter.

The optimum length of labelling was studied. Individual 35 mm culture dishes were each rendered "quiescent" in 1% FBS for a constant time of 24 hours. This was followed by a constant 24 hour unlabelled stimulation period with 10% FBS. At this time, ^{3}H-thymidine was added to the medium as stimulation continued. The labelling time was varied from three hours to forty-eight hours (Figure 4), with each time represented by four replicates. Incorporation of ^{3}H-thymidine appeared to increase linearly from three to forty-eight hours of labelling. Therefore, the non-neuronal cells incorporate more ^{3}H-thymidine the longer they are exposed

to it. A twenty-four hour labelling time was chosen as a convenient time which was employed in future assays.

Summary of Optimum Cell Culture and Bioassay Conditions

A summary of the optimum cell culture and bioassay conditions can be found in Table II. Collagen-coated, rather than uncoated culture dishes promoted faster, more extensive non-neuronal cell growth, particularly for primary cells. The most growth responsive cell culture ages were the second and third subcultures. Complete growth medium containing insulin, sodium selenate, transferrin, putrescine, and progesterone in addition to α-MEM, FBS, glucose, and antibiotic/antimycotic allowed the best non-neuronal cell growth. Medium supplemented with 1% FBS, as opposed to 0.1% FBS, used for rendering the cells quiescent and as a base for the test factors was found to give healthier non-neuronal cells. The most effective bioassay conditions included a minimum 14.5 hour "quiescence" time (24 hours was adopted), a 6 hour stimulation time, and a 24 hour ^{3}H-thymidine labelling time.

Cell Culture Composition

An attempt was made to characterize the chick embryonic brain cell composition using the technique of immunohistochemistry. The cultured cells utilized in this research had the distinctive cobblestone morphology attributed to confluent astroblasts (astrocyte precursors) previously observed in this laboratory (Carlone et. al., 1988) and by others in the chick embryo (McCarthy and Partlow, 1976) and new born mouse (Sensenbrenner and Mandel, 1974). Astrocytes are characterized by intermediate glial filaments in their cytoplasm; these fibres consist of glial fibrillary acid protein (GFAP) and antibodies against it may be used to

Table II. Summary of Optimum Cell Culture and Bioassay Conditions

Cell Culture Conditions	
collagen-coated vs. uncoated culture dishes	collagen-coated
cell subculture	2^{nd} or 3^{rd}
complete vs. incomplete medium	complete
1% vs. 0.1% FBS for assay control	1%
Bio-Assay Conditions	
"quiescence" time	minimum 14.5 hr (24 hr used)
stimulation time	6 hr
^{3}H-thymidine labelling time	24 hr

identify astrocytes. Subcultured confluent non-neuronal chick embryo brain cells have been found to be composed of >90% GFAP positive cells when tested one week after plating (Carlone et. al., 1988). In this study, preliminary evidence indicated that primary non-neuronal cells tested 4 days after plating while cells were subconfluent, consisted of 6.63% GFAP positive cells (several GFAP positive non-neuronal cells showing clear cytoplasmic staining are apparent in Figure 5). These results represent the mean percentage of GFAP positive cells in two fields of view with a total of 581 cells counted. These apparently conflicting results suggest that the cells used in this research were composed primarily of astroblasts; these precursors do not stain for GFAP (Pixley *et al.*, 1984).

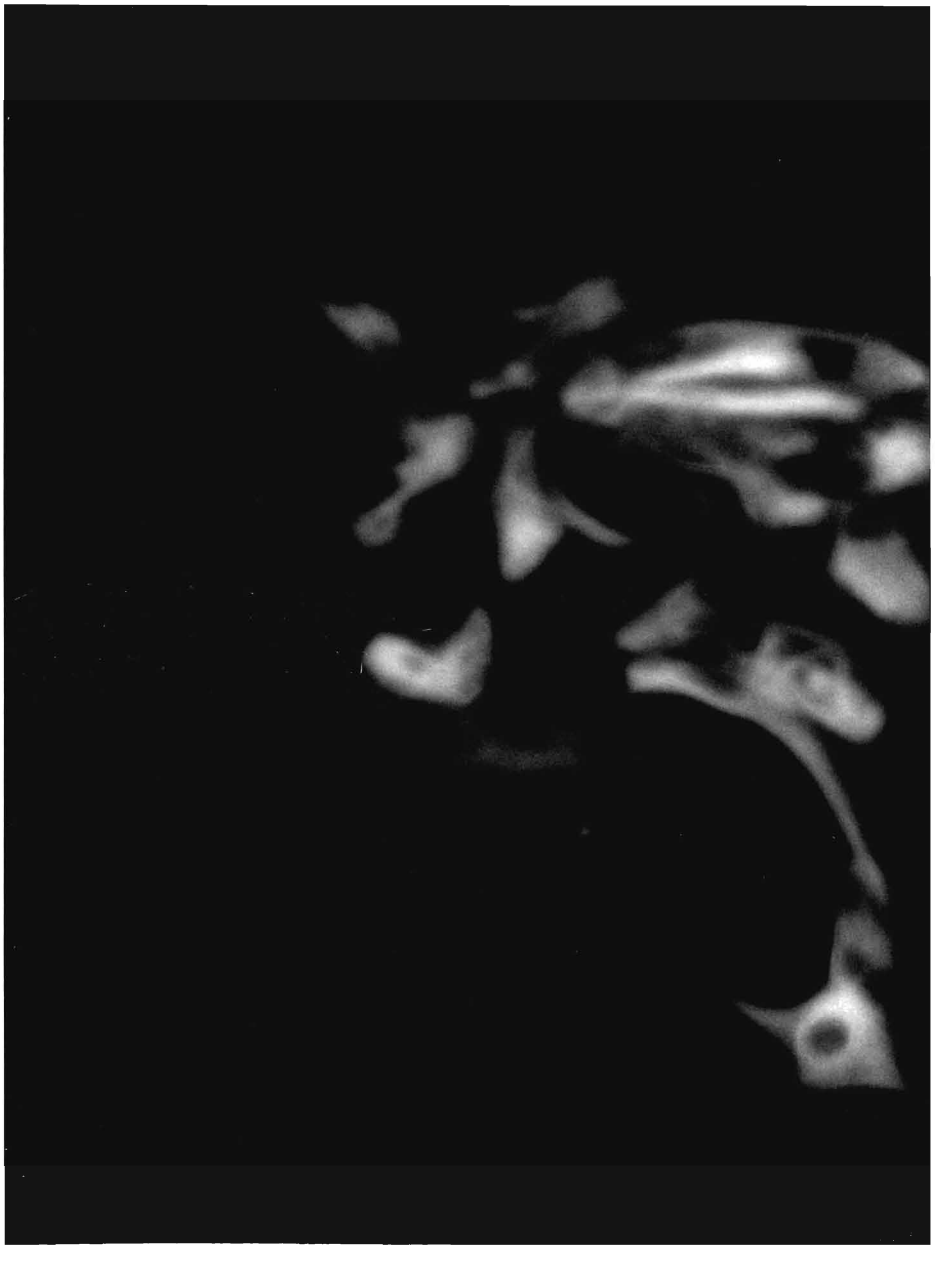
Isolation and Purification of Chick Brain Growth Factor

A. Changes to the isolation and purification procedure

The isolation of chick brain growth factor (CBGF) has been described previously (Carlone et. al., 1985; 1987). The brains of ten to twenty dozen eighteen day chick embryos are removed, homogenized, and centrifuged at 100,000 x G. The supernatant undergoes ultrafiltration by passing it through a series of Amicon filters of decreasing porosity: a PM-30 membrane of nominal molecular weight cut-off 30,000 Daltons (Da.) and a YM-2 membrane of nominal molecular weight cut-off 2,000 Da. In each case, the filtrate is used for the proceeding step. At this point, the isolation procedure employed in this research diverges from that outlined by Carlone et. al. (1985, 1987). Previously, the YM-2 filtrate was applied to a UM-05 membrane (nominal molecular weight cut-off of 500 Da.) and the retentate was collected. Amicon ceased manufacture of this membrane, made of a blend of polyelectrolytes, and replaced it with a cellulose YC-05

Figure 5. Glial Fibrillary Acidic Protein (GFAP) Immunofluorescence of Cultured Primary 10-day Chick Embryo Brain Non-neuronal Cells.

Immunofluorescence processing of the cells is described in the text. x250.



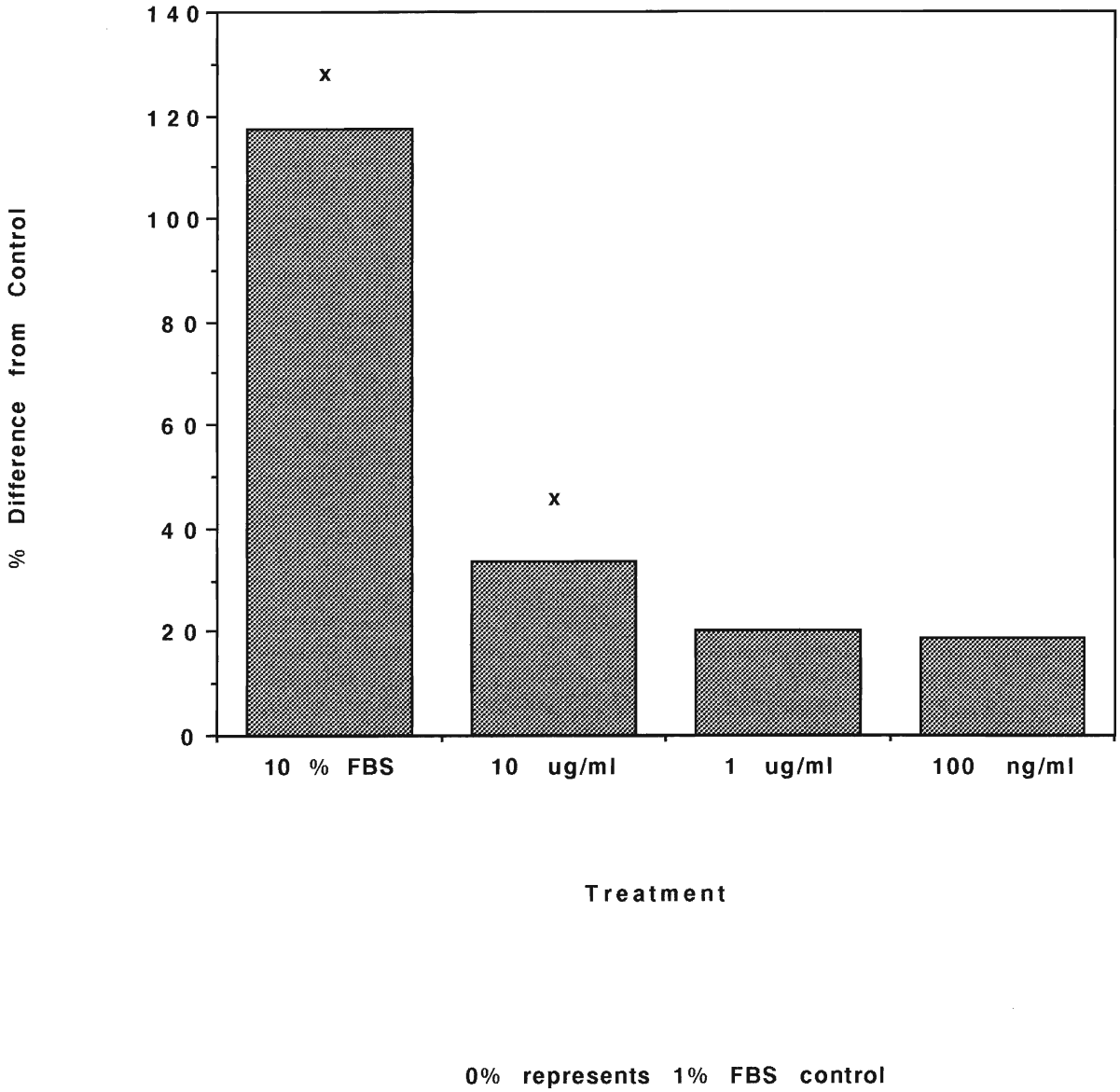
membrane, having a molecular weight cut-off of 300 to 400 Da. Prior to this study, several attempts were made to isolate an active fraction from the YC-05 retentate, but these efforts failed. The YM-2 filtrate was also applied to a Biogel P2 size exclusion column (with molecular weight cut-off of 2,000 Da.); however, no separation of the material resulted, with only one peak eluting. Consequently, in this research, the YM-2 filtrate was applied to a Biogel P6 size exclusion column (with molecular weight cut-off of 6,000 Da.) from which a biologically active fraction was collected. Use of a DEAE anion exchange column was discontinued, as it had been in a previous study (Seifried, honours thesis, 1988). Anion exchange high performance liquid chromatography was used to separate the P6 fraction, but difficulties in testing the resultant fractions caused this technique to be abandoned. The subsequent purification step was reverse phase high performance liquid chromatography (rp-HPLC). The CBGF isolation procedure was undertaken four times during the course of this work.

B. Biological activity of ultrafiltration fractions

After the brain homogenate was centrifuged at 100,000xG, it was passed through an Amicon PM-30 membrane having a molecular weight cut off of 30,000 Da. The biological activity of the PM-30 filtrate was determined using the non-neuronal cell bioassay. In Figure 6, the effects of various concentrations of the PM-30 filtrate, as well as the 10% FBS control, on ^{3}H-thymidine incorporation in third passage non-neuronal cells are given. As can be seen, 10 µg/ml PM-30 filtrate significantly stimulates ^{3}H-thymidine uptake in these cells, causing a 33.8% increase over the control. A concentration dependence of the PM-30 filtrate on cell growth was displayed since the lower concentrations (1 µg/ml and 100 ng/ml) elicit decreasing amounts of growth stimulation. The stimulation seen at 1 µg/ml and 100 ng/ml PM-30 filtrate was not statistically significant. As

Figure 6. Effect of PM-30 Filtrate on 3H-thymidine Incorporation in Cultured Chick Embryo Brain Non-neuronal Cells.

Data are presented as percent difference from the 1% FBS control for each of the PM-30 filtrate concentrations tested and the 10% FBS control. Statistically significant differences ($p \leq 0.05$) are represented by an "x" over the corresponding bar on the graph. n = 6 for each PM-30 filtrate test concentration; n = 3 for the 1% and 10% FBS controls. Third subculture cells were used. (FBS = fetal bovine serum)



0% represents 1% FBS control

was expected, 10% FBS gave a significant increase in cell growth with a 117.2 % difference over control.

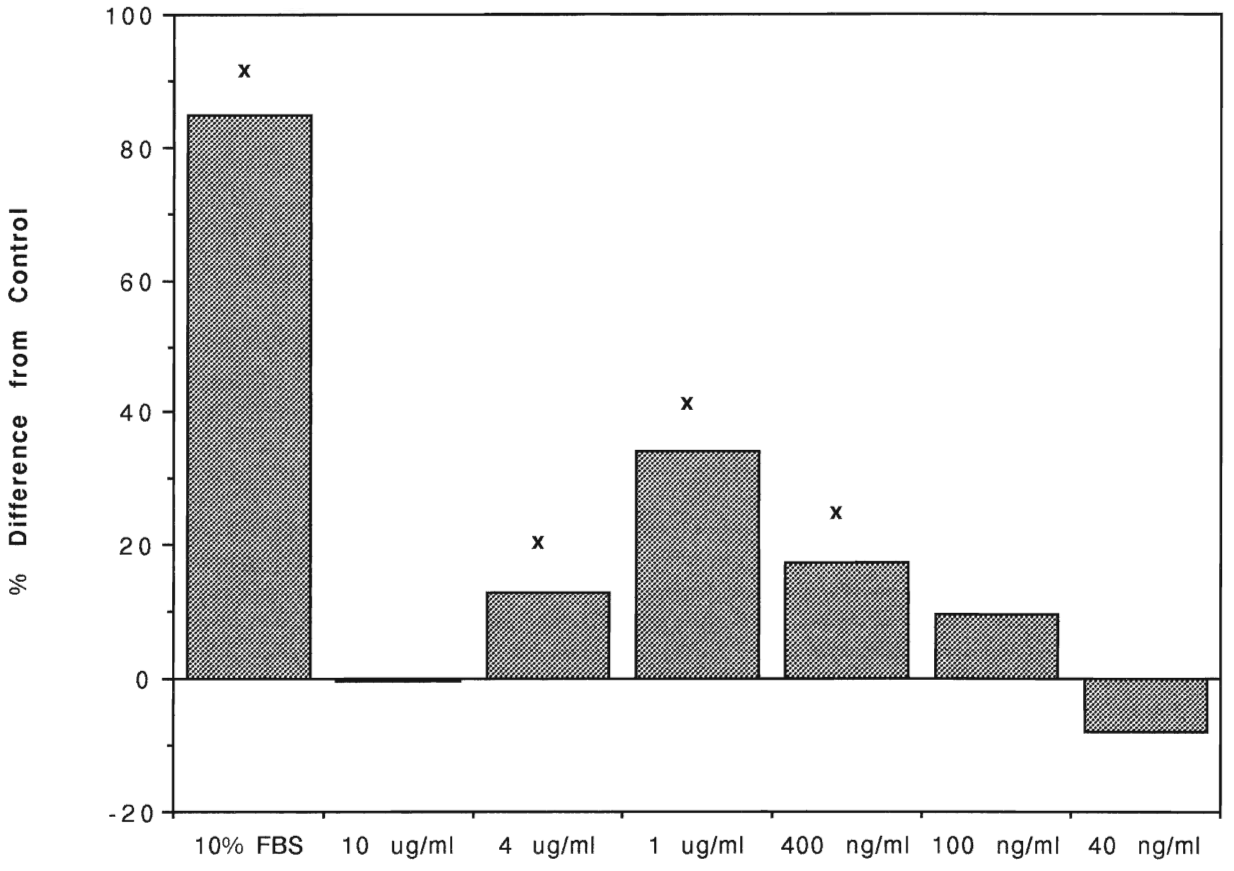
Once it had gone through the PM-30 membrane, the filtrate was passed through an Amicon YM-2 membrane having a molecular weight cut-off of approximately 2,000 Da. A compilation of data from two bioassays of the YM-2 filtrate is represented in Figure 7; YM-2 filtrate concentrations of 4 µg/ml, 400 ng/ml, and 40 ng/ml were tested on first subculture cells in one twenty-four well plate, while concentrations of 10 µg/ml, 1 µg/ml, and 100 ng/ml were tested on second subculture cells in the second plate. As can be visualized in Figure 7, the YM-2 filtrate was effective at causing an increase in ^{3}H-thymidine uptake, with maximum stimulation at 1 µg/ml. This treatment resulted in a statistically significant increase of 34.0% over the control. Once again growth stimulation in these cells was dependent on the YM-2 filtrate concentration used, with a bell-shaped concentration dependence apparent. Both high (10 µg/ml) and low (10 ng/ml and 40 ng/ml) concentrations of the YM-2 filtrate had no significant effect on cell growth, with intermediary concentrations (4 µg/ml and 400 ng/ml) having a diminished significant stimulatory effect. The value of 84.9% increase over control given for 10% FBS is the mean determined from the 80.7% increase of the first assay (n=4) and the 89.0% increase of the second assay (n=4).

C. Elution profile and biological activity of biogel P6 fractions

Following ultrafiltration, the YM-2 filtrate was lyophilized, reconstituted in a small volume of distilled water (approximately 1 ml) and applied to a Biogel P6 size exclusion column (molecular weight cut-off of 6,000 Da.). The fractions were collected at ten minute intervals at a flow rate of 0.165 ml/min with a resultant fraction volume of 1.65 ml. The ultraviolet (UV) absorbance of these fractions was measured at 260 nm

Figure 7. Effect of YM-2 Filtrate on 3H-thymidine Incorporation in Cultured Chick Embryo Brain Non-neuronal Cells.

Data are presented as percent difference from the 1% FBS control for each of the YM-2 filtrate concentrations tested and the 10% FBS control. Statistically significant differences ($p \leq 0.05$) are represented by an "x" over the corresponding bar on the graph. Data have been combined from two YM-2 filtrate bioassays. YM-2 filtrate concentrations of 4 µg/ml, 400 ng/ml, and 40 ng/ml were tested on first subculture cells on one 24 well plate, while concentrations of 10 µg/ml, 1 µg/ml, and 100 ng/ml were tested on second subculture cells on a second plate; the value for 10% FBS is the mean determined from the two plates. For each plate, n = 5 for each YM-2 filtrate test concentration and the 1% FBS control; n = 4 for the 10% FBS control. (FBS = fetal bovine serum)



0% represents 1% control

(nucleotide absorbance) and 280 nm (tyrosine and tryptophan absorbances) and was plotted as a function of test tube fraction number. Over the course of this research, four distinct CBGF preparations were undertaken, and similar Biogel P6 elution profiles resulted. Figure 8 provides a representative elution profile from the third CBGF preparation. As can be seen in Figure 8, three poorly resolved UV absorbing peaks were obtained. The first peak, P6-2 (tube numbers 17 to 27), contained relatively equal amounts of components absorbing at 260 nm and 280 nm. The second peak, P6-3 (tube numbers 28 to 31), was comprised of greater 260 nm than 280 nm absorbing components, as was also the case for the smaller third peak, P6-4 (tube numbers 32 to 41).

The biological activity of various concentrations of each of these three fractions on non-neuronal cells was determined. From Figure 9a and b, it can be seen that fraction P6-2 was not mitogenic. However, at 3 μg/ml this fraction was inhibitory to growth, causing a statistically significant decrease of 55.7% in ^{3}H-thymidine incorporation. The fraction also caused a slight decline in growth at 30 ng/ml (significant in the first bioassay). In Figure 9a, 10% FBS effected a 65.1% increase in growth, while in Figure 9b a 117.0% increase was found. Variations in the non-neuronal cells' response to this known growth stimulus are also reflected in the cells' ability to respond to test factors. Thus, the cells of the bio-assay represented in Figure 9a were less responsive to 10% FBS than the first subculture cells of the assay depicted in Figure 9b; concomitantly, this decrease is mirrored in Figure 9a cells treated with 300 ng/ml and 30 ng/ml P6-2 compared to similarly treated cells in Figure 9b.

The graph of Figure 10 represents a compilation of data from two bioassays of P6-3 on second passage non-neuronal cells; P6-3 concentrations of 3 μg/ml, 1 μg/ml, and 300 ng/ml were assayed on one twenty-four well plate, while P6-3 concentrations of 100 ng/ml, 30 ng/ml, and 3 ng/ml were assayed on the second plate. The value of 143.3%

Figure 8. Biogel P6 Elution Profile of YM-2 Filtrate Fractionation.

A Biogel P6 size exclusion column (nominal molecular weight cut-off of 6,000 Da.) was used to separate the concentrated YM-2 filtrate into three peaks labelled P6-2 (fractions 17-27: red), P6-3 (fractions 28-31: blue), and P6-4 (fractions 32-41: yellow). Fractions were eluted with 0.05 M ammonium acetate and collected at 10 minute intervals, yielding 1.65 ml fraction volumes. Samples from the individual fractions were diluted 100x and UV absorbances were measured at 280 nm (dotted line) and 260 nm (solid line).

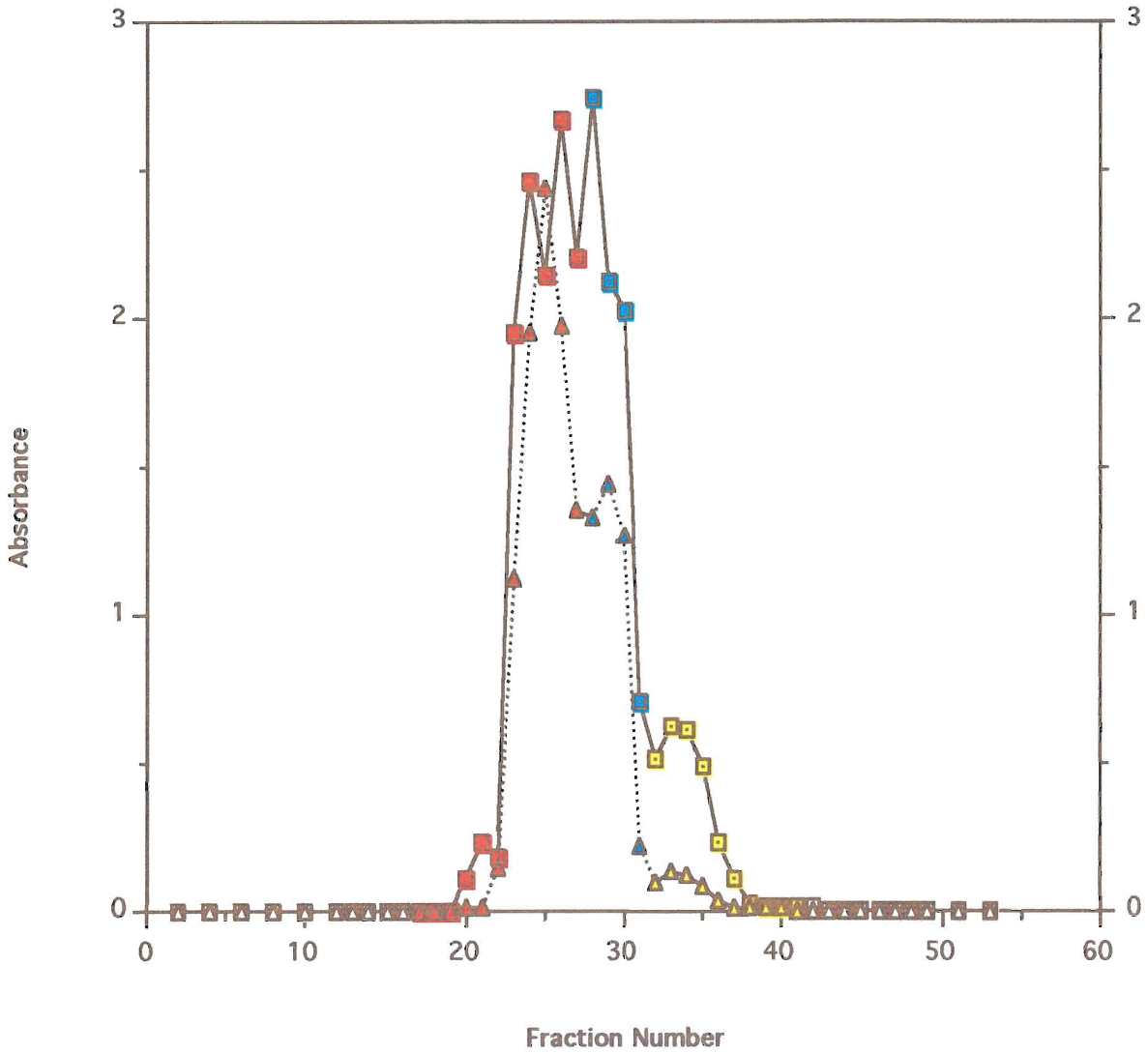
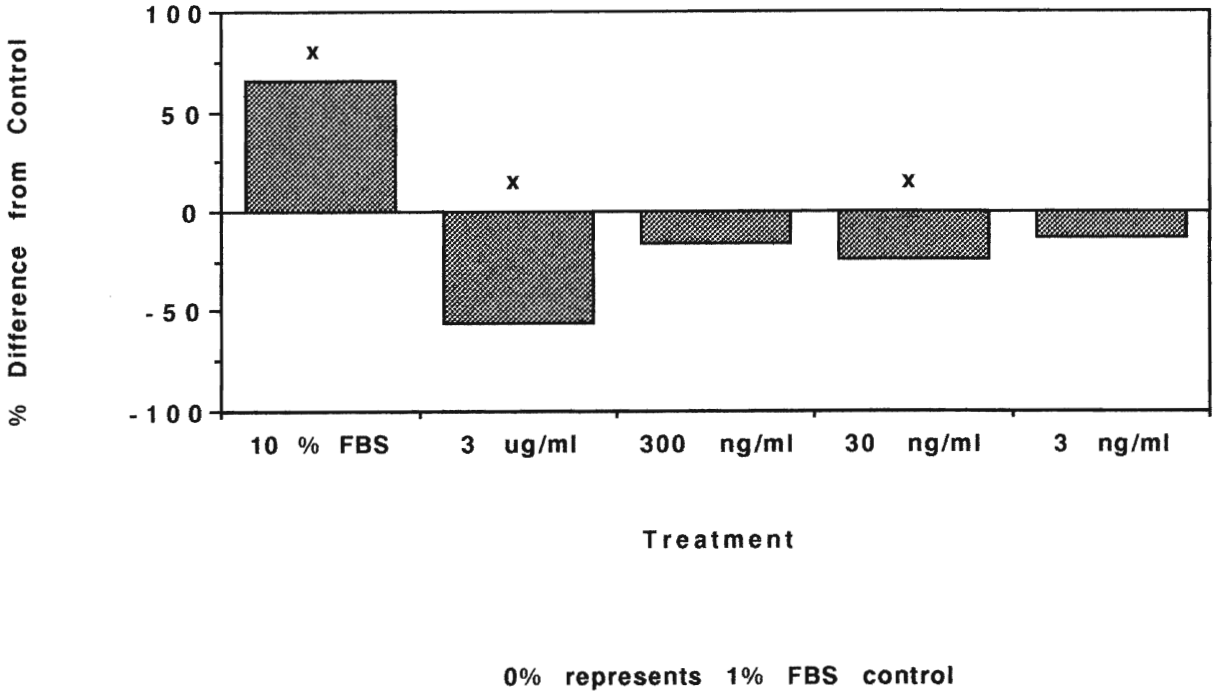

Absorbance
Fraction Number
0
1
2
3
0
10
20
30
40
50
60

Figure 9. Effect of P6-2 on 3H-thymidine Incorporation in Cultured Chick Embryo Brain Non-neuronal Cells.

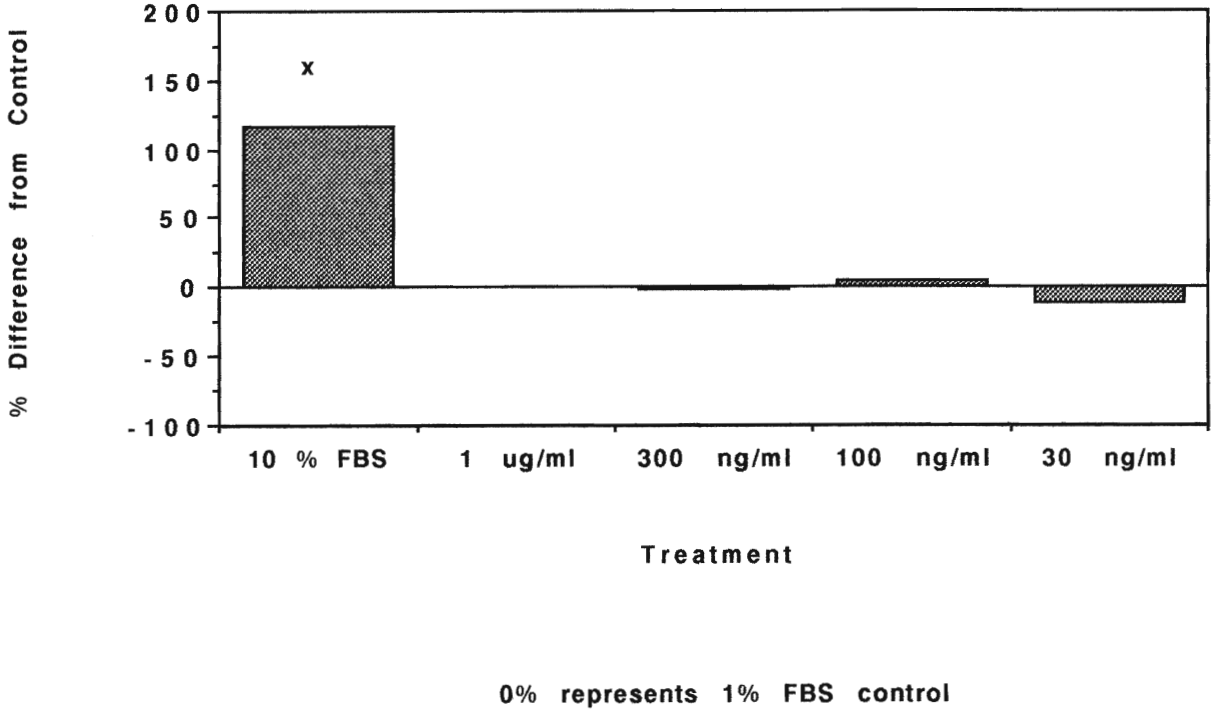
Data are presented as percent difference from the 1% FBS control for each of the P6-2 concentrations tested and the 10% FBS control. Statistically significant differences ($p \leq 0.05$) are represented by an "x" over the corresponding bar on the graph. n = 4 for each P6-2 test concentration and the 1% and 10% FBS controls.
(FBS = fetal bovine serum)
A: P6-2 concentrations tested: 3 µg/ml, 300 ng/ml, 30 ng/ml, and 3 ng/ml. First subculture cells were used.
B: P6-2 concentrations tested: 1 µg/ml, 300 ng/ml, 100 ng/ml, and 30 ng/ml. First subculture cells were used.

A



0% represents 1% FBS control

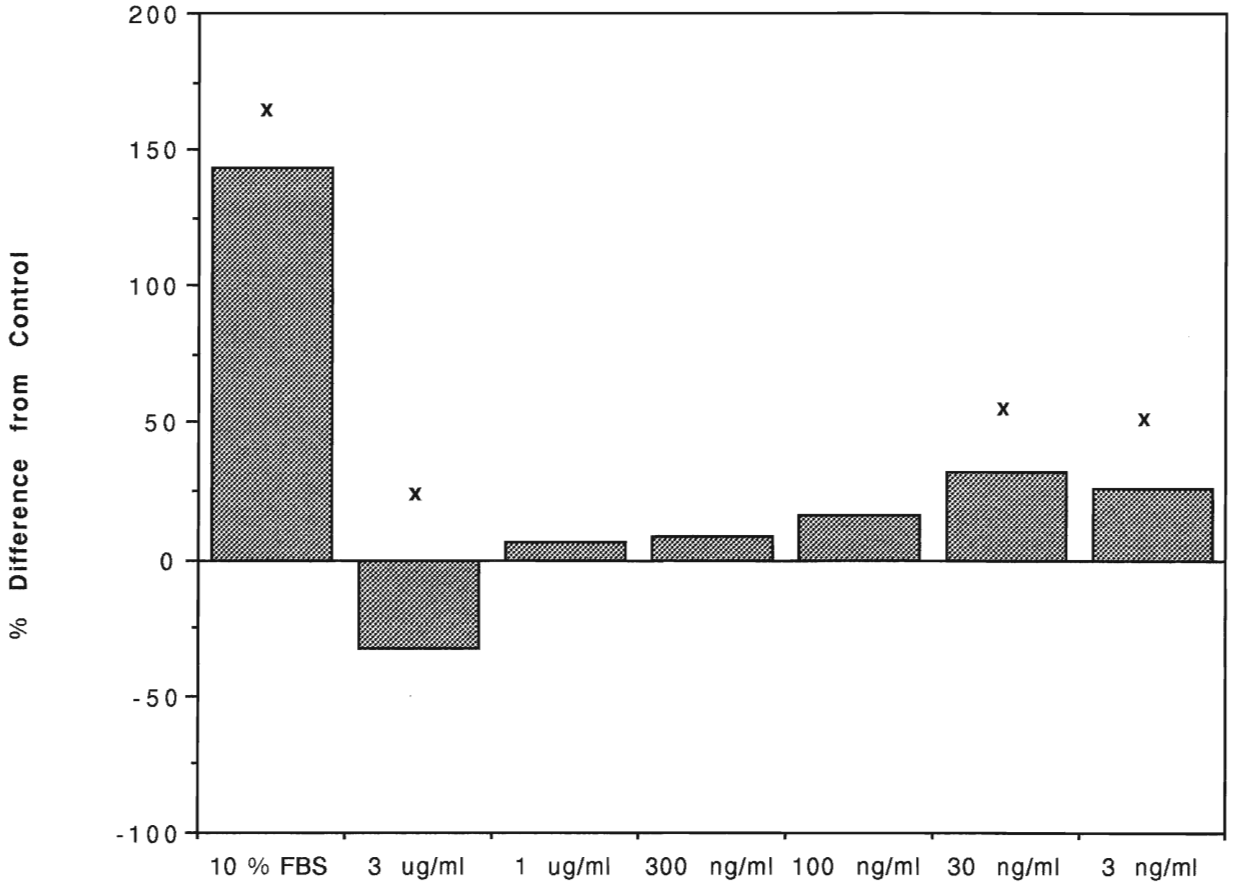
B



0% represents 1% FBS control

Figure 10. Effect of P6-3 on 3H-thymidine Incorporation in Cultured Chick Embryo Brain Non-neuronal Cells.

Data are presented as percent difference from the 1% FBS control for each of the P6-3 concentrations tested and the 10% FBS control. Statistically significant differences are represented by an "x" over the corresponding bar on the graph. Data have been combined from two P6-3 bioassays on second subculture cells in which P6-3 test concentrations of 3 μg/ml, 1 μg/ml, and 300 ng/ml were tested on one culture plate and 100 ng/ml, 30 ng/ml, and 3 ng/ml were tested on the second culture plate; the value for 10% FBS is the mean determined from the two plates. For each plate, n = 6 for each P6-3 test concentration; n = 3 for the 1% and 10% FBS controls. (FBS = fetal bovine serum)



0% represents 1% FBS control

increase over control given for 10% FBS is the mean determined from the 172.8% increase of the first assay (n=4) and the 113.7% increase of the second assay (n=4). The biological activity of fraction P6-3 displayed a concentration dependence over the range 3 µg/ml to 3 ng/ml with high concentrations of this material being inhibitory and lower concentrations being stimulatory to cell growth. At 3 µg/ml, P6-3 caused a statistically significant 32.2% inhibition of ^{3}H-thymidine incorporation in these non-neuronal cells. This fraction produced no effect at 1 µg/ml, 300 ng/ml and 100 ng/ml. At a concentration of 30 ng/ml, fraction P6-3 gave a significant 32.3% increase in cell proliferation. At 3 ng/ml, the increase in cell growth had diminished to 26.2%, but was still a statistically significant effect.

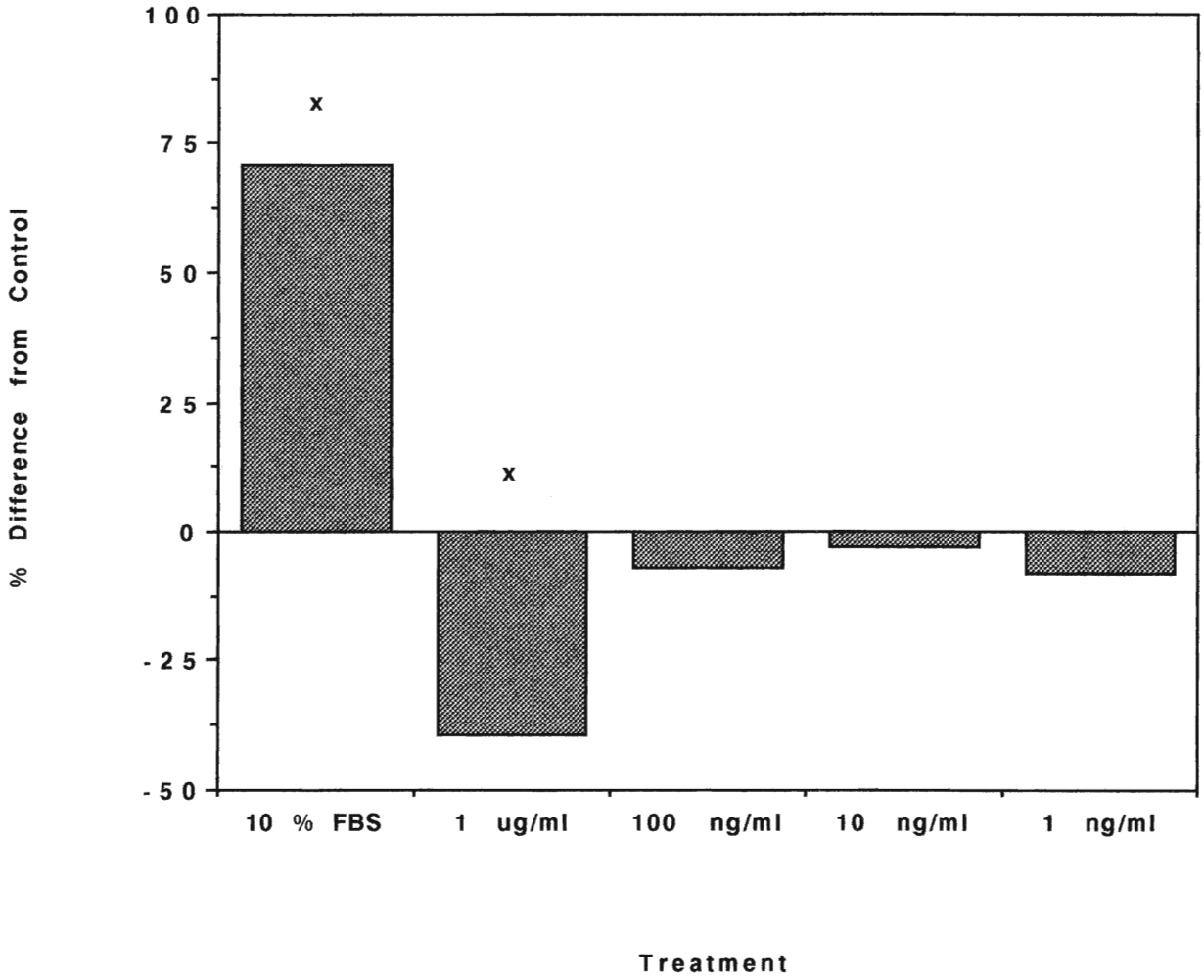
Fraction P6-4 was found not to cause cell proliferation and was in fact inhibitory at high concentrations. From Figure 11, one can see that P6-4 caused a statistically significant 39.2% decrease in growth as compared to the control when tested at 1 µg/ml. No effect was generated when the cells were treated with 100 ng/ml, 10 ng/ml or 1 ng/ml of P6-4. The 10% FBS caused a significantly stimulatory response of 70.8%.

D. Elution profile and biological activity of anion exchange HPLC fractions

Previous studies had indicated that the mitogenic activity resided in a polar molecule. This was evidenced by the elution of HPLC fractions under an isocratic solvent system of H_2O with 0.05% trifluoroacetic acid (TFA) or 0.1% acetonitrile (CH_3CN) in H_2O with 0.05% TFA (Seifried, honours thesis, 1988). For this reason, it was thought that fractionation of the material in the P6-3 fraction should be based on charge. With this intention, anion exchange HPLC was attempted. The elutions profiles at 280 nm, 256 nm and 229 nm obtained from a separation of fraction P6-3

Figure 11. Effect of P6-4 on ^{3}H-thymidine Incorporation in Cultured Chick Embryo Brain Non-neuronal Cells.

Data are presented as percent difference from the 1% FBS control for each of the P6-4 concentrations tested and the 10% FBS control. Statistically significant differences ($p \leq 0.05$) are represented by an "x" over the corresponding bar on the graph. n = 4 for each P6-4 test concentration and the 1% and 10% FBS controls. Third subculture cells were used. (FBS = fetal bovine serum)



0% represents 1% FBS control

are presented in Appendix A. Approximately seven peaks were apparent. Each of the seven fractions identified in the elution profile at 256 nm were tested for their ability to stimulate non-neuronal cell growth. Since the column was run under a gradient employing solutions with high concentrations of KH_2PO_4 and KCl (0.1% 0.007M KH_2PO_4 and 0.007M KCl pH 4.0 to 99.9% 0.25M KH_2PO_4 and 0.50M KCl pH 5.0 in 20 minutes), the eluted samples also contained large amounts of these salts. Consequently, it was necessary to test a salt control corresponding to the KH_2PO_4 and KCl content of each fraction at every concentration examined. Appendix A contains the results of such assays. Data are presented in bar graph form in which the mean incorporation of ^{3}H-thymidine in cpm is given for each treatment. Thus, the value for ^{3}H-thymidine incorporation for the 1% FBS control is also given, since these results are not presented as a percentage from the control. To accommodate every test condition for four concentrations of each anion exchange HPLC fraction, two twenty-four well bioassay plates were used per fraction.

When compared to their corresponding salt controls, it was found that anion exchange HPLC fraction #2 at 200 ng/ml and 2 ng/ml and fraction #5 at 30 ng/ml were biologically active. These results were confounded by the fact that the salt controls were found to cause more ^{3}H-thymidine incorporation than their corresponding test factor in fraction #2 at 20 ng/ml and 0.2 ng/ml. In these instances, the salt controls were found also to be mitogenic compared to the 1% FBS control. Furthermore, high concentrations of these salts were found to be inhibitory to non-neuronal cell growth. Such variable results and conditions were not deemed suitable for accurately determining the mitogenic status of these fractions. Moreover, the high KH_2PO_4 and KCl concentrations made it difficult to estimate the protein content of the fractions using the Lowry protein assay. In fact, upon addition of the Folin reagent, a white precipitate formed. This was removed following centrifugation, however

potential interference with the reaction itself or in the absorbance readings rendered these protein determinations unreliable. Due to these severe salt effects, use of anion exchange HPLC as a purification step in this procedure was discontinued.

E. Elution profile and biological activity of reverse phase HPLC fractions

As a means to further purify the biologically active material, fraction P6-3 was lyophilized, reconstituted in a small volume of distilled water, and subjected to C_{18} reverse phase high performance liquid chromatography (rp-HPLC). The elution was accomplished under a relatively steep gradient of 0.1% to 99.9% CH_3CN in H_2O with 0.05% TFA in twenty minutes; an initial two minute period of isocratic 0.1% H_2O with 0.05% TFA followed by the gradient was also effective. A typical reverse phase HPLC elution profile recorded at 280 nm is given in Figure 12. As can be seen, eleven ultraviolet absorbing peaks were observed. Figure 13 shows the rp-HPLC elution profile recorded at 256 nm, a wavelength typically associated with nucleic acid absorbance. While it is not possible to directly compare the rp-HPLC elution profiles of Figures 12 and 13 since different concentrations of fraction P6-3 were loaded onto the column, an examination of relative peak heights is possible. Thus, it is apparent that peak 6 appeared to have increased proportionately more than the others. This is particularly evident when comparing the similar peak heights of peaks 4 and 6 in Figure 12 to the notable increase in the height of peak 6 in Figure 13. Although the height of peak 1 is smaller in Figure 13 than in Figure 12, the height of this peak at 280nm was inconsistent throughout the numerous rp-HPLC elutions which were performed. Each of the eleven peaks was tested in the bioassay for its effect on non-neuronal cell

Figure 12. RP-HPLC Elution Profile of P6-3 Fractionation Monitored at 280 nm.

A μBondapak C_{18} column was used to separate the concentrated Biogel P6-3 fraction into eleven peaks, which were individually collected. The fractions were eluted with a gradient of 0.1% to 99.9% CH_3CN in H_2O with 0.05% TFA in 20 minutes. (RP-HPLC = reversed phase high-performance liquid chromatography; TFA = trifluoroacetic acid)

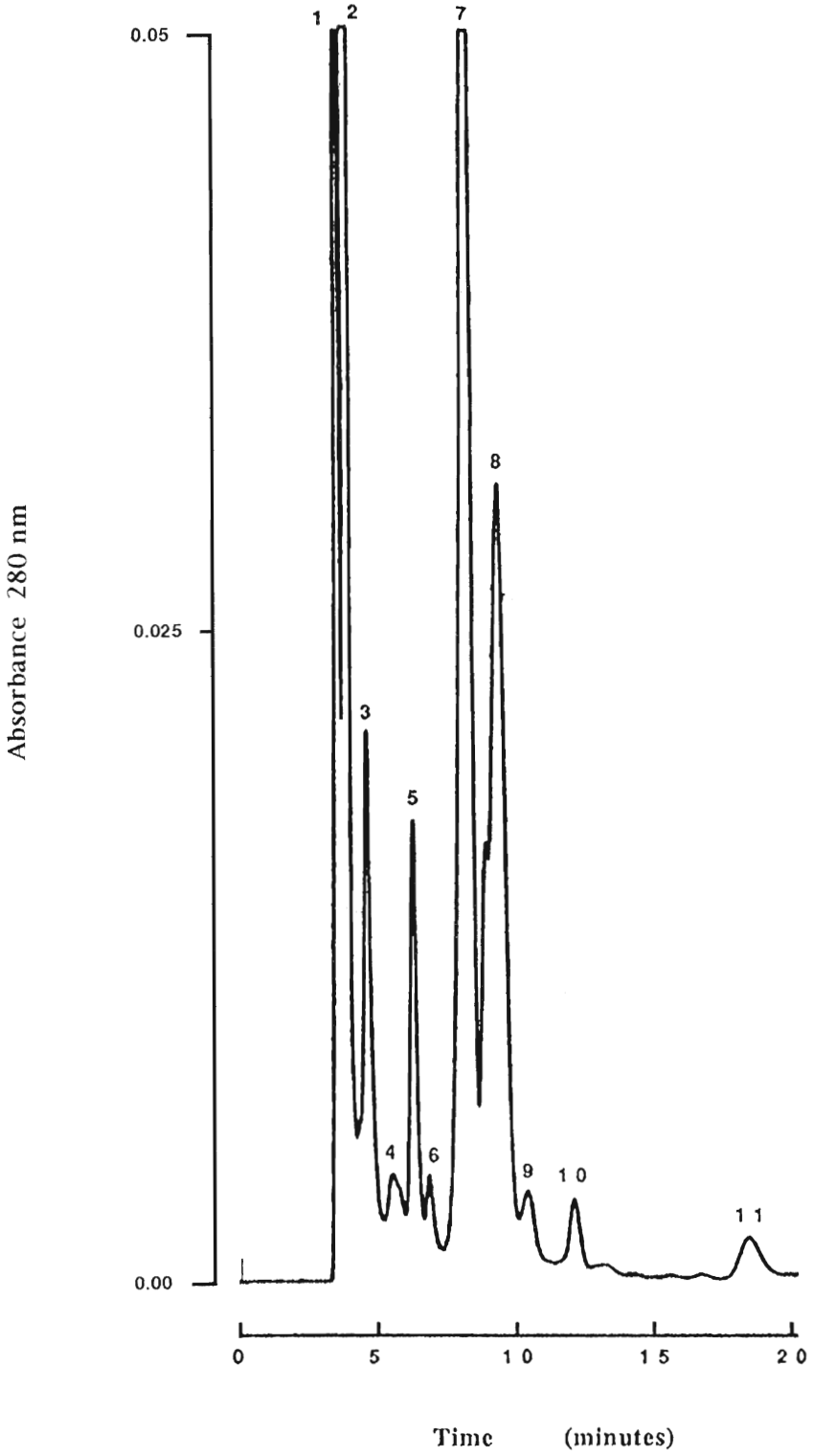
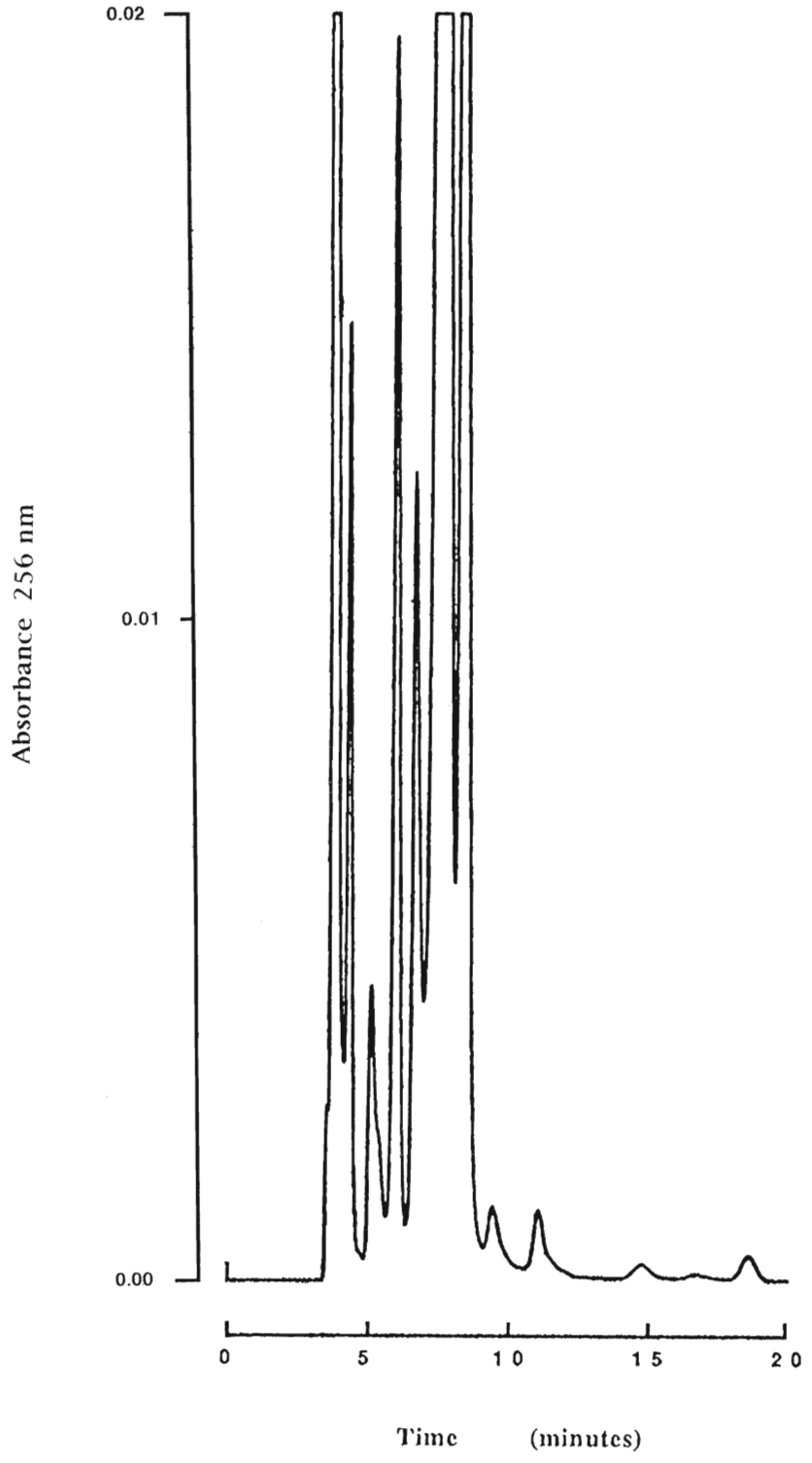

Absorbance 280 nm
0.05
0.025
0.00
1
2
3
4
5
6
7
8
9
10
11
0
5
10
15
20
Time (minutes)

Figure 13. RP-HPLC Elution Profile of P6-3 Fractionation Monitored at 256 nm.

A μBondapak C_{18} column was used to separate the concentrated Biogel P6-3 fraction. The peaks were eluted with a gradient of 0.1% to 99.9% CH_3CN in H_2O with 0.05% TFA in 20 minutes. (RP-HPLC = reversed phase high-performance liquid chromatography; TFA = trifluoroacetic acid)


0.02
0.01
0.00
Absorbance 256 nm
0
5
10
15
20
Time (minutes)

growth. Three concentrations of every fraction were examined: 100 ng/ml, 10 ng/ml and 1 ng/ml.

In Figure 14a, it can be seen that rp-HPLC fraction #1 at 10 ng/ml caused a significant 23.7% increase in ^{3}H-thymidine incorporation over the 1% FBS control. While it was stimulatory at this concentration, this was not the case at 100 ng/ml and 1 ng/ml where no effect was apparent. A dose-dependent response was observed over the three tested concentrations. In this assay, 10% FBS significantly stimulated a 118.5% increase in ^{3}H-thymidine uptake in these cells.

Figure 14b depicts the results found in the bioassay of rp-HPLC fraction #2. This material had no effect on ^{3}H-thymidine incorporation in the non-neuronal cells at any of the three concentrations tested. The 10% FBS control registered a statistically significant increase in cell growth of 66.1%.

Like rp-HPLC fraction #2, rp-HPLC fraction #3 did not cause a change in ^{3}H-thymidine uptake when compared to control cells treated with 1% FBS (Figure 14c). However, 10% FBS affected a significant 83.8% stimulation of non-neuronal cell growth in this assay.

In contrast, rp-HPLC fraction #4 was actually inhibitory to non-neuronal cell growth causing a statistically significant 21.3% decrease in ^{3}H-thymidine uptake when tested at 100 ng/ml (Figure 15a). This fraction caused no effect on cell growth when administered at 10 ng/ml or 1 ng/ml. Treatment with 10% FBS resulted in significant 96.5% stimulation of cell proliferation.

Similarly, rp-HPLC fraction #5 also caused significant inhibition of non-neuronal cell proliferation (Figure 15b). Exposing the cells to 100 ng/ml this fraction resulted in an even greater decrease in ^{3}H-thymidine incorporation (-55.4%) than that caused by rp-HPLC fraction #4. No effect on cell growth was seen when this fraction was tested at

Figure 14. Effect of rp-HPLC Fractions #1, 2, and 3 on ^{3}H-thymidine Incorporation in Cultured Chick Embryo Brain Non-neuronal Cells.

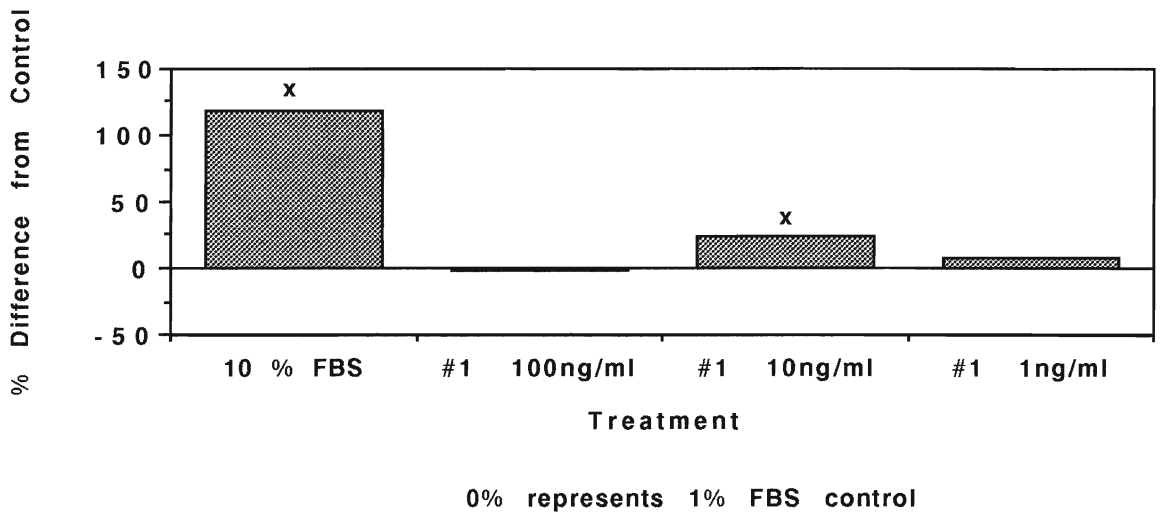
Data are presented as percent difference from the 1% FBS control for each of the rp-HPLC fraction concentrations tested and the 10% FBS control. Statistically significant differences ($p \leq 0.05$) are represented by an "x" over the corresponding bar on the graph. n = 5 for each rp-HPLC fraction test concentration and the 1% FBS control; n = 4 for the 10% FBS control. First subculture cells were used. (rp-HPLC = reversed-phase high-performance liquid chromatography; FBS = fetal bovine serum)

A: Results for rp-HPLC fraction #1.

B: Results for rp-HPLC fraction #2.

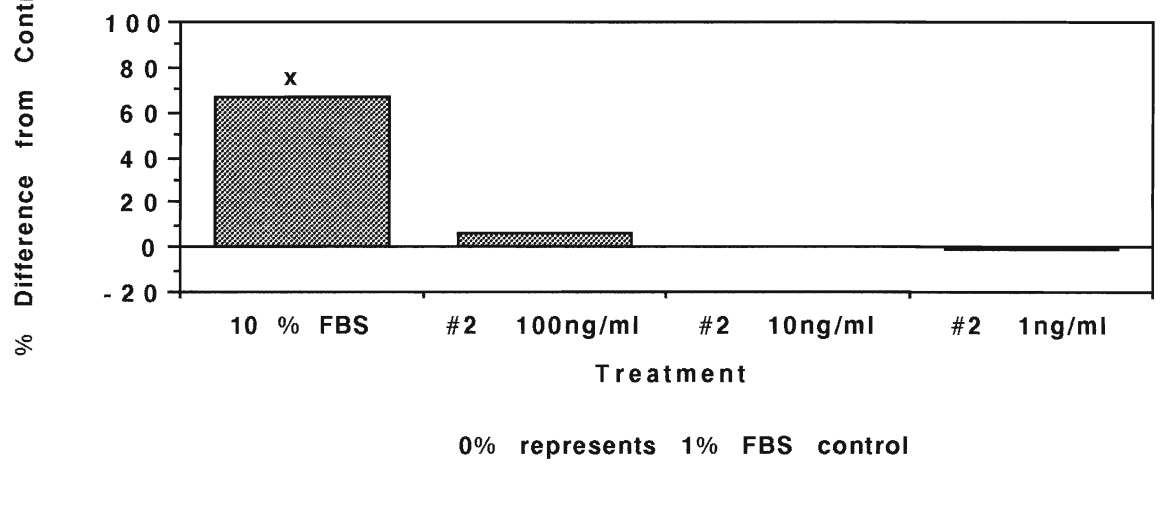
C: Results for rp-HPLC fraction #3.

A



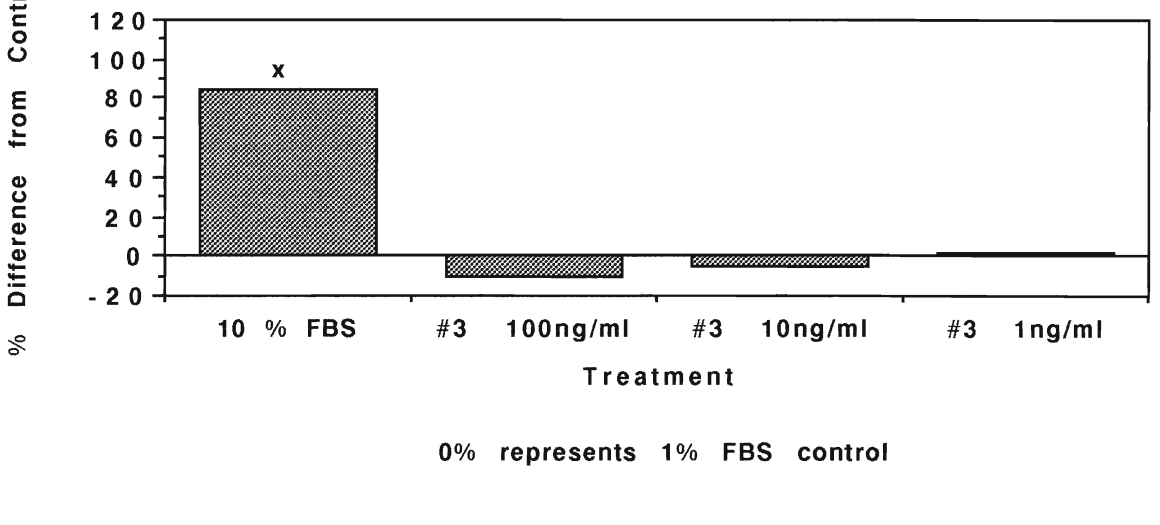
0% represents 1% FBS control

B



0% represents 1% FBS control

C



0% represents 1% FBS control

Figure 15. Effect of rp-HPLC Fractions #4, 5, and 6 on ^{3}H-thymidine Incorporation in Cultured Chick Embryo Brain Non-neuronal Cells.

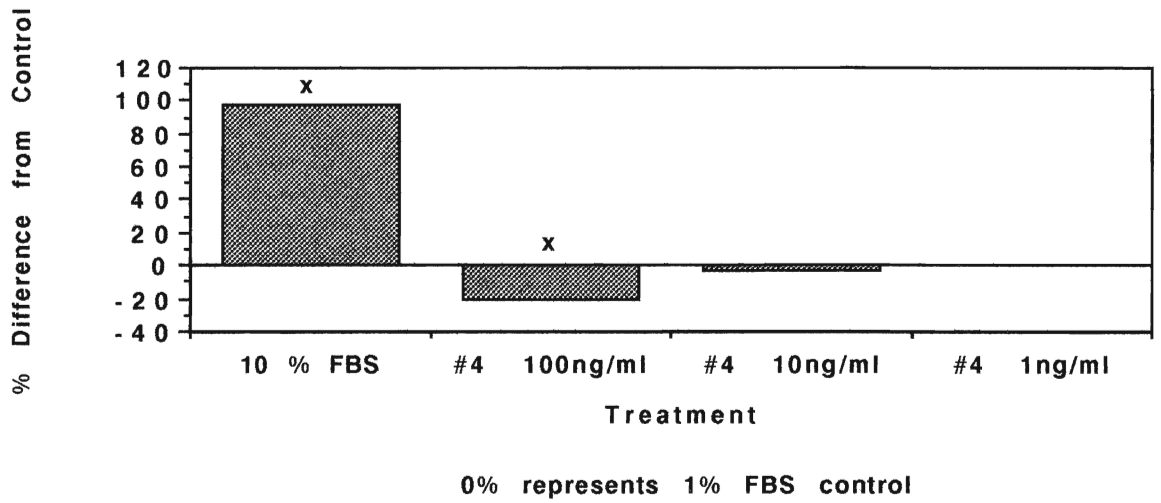
Data are presented as percent difference from the 1% FBS control for each of the rp-HPLC fraction concentrations tested and the 10% FBS control. Statistically significant differences ($p \leq 0.05$) are represented by an "x" over the corresponding bar on the graph. n = 5 for each rp-HPLC fraction test concentration and the 1% FBS control; n = 4 for the 10% FBS control. (rp-HPLC = reversed-phase high-performance liquid chromatography; FBS = fetal bovine serum)

A: Results for rp-HPLC fraction #4. First subculture cells were used.

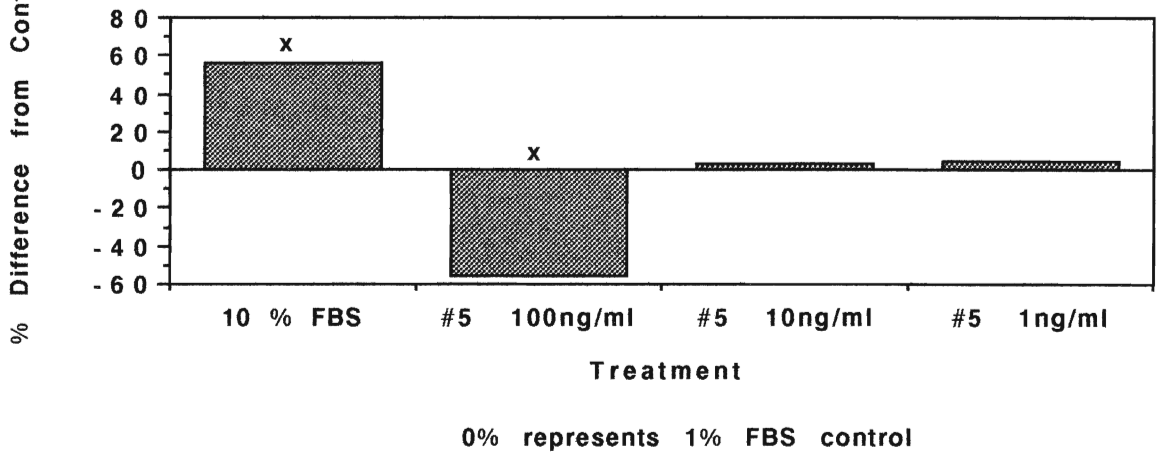
B: Results for rp-HPLC fraction #5. Second subculture cells were used.

C: Results for rp-HPLC fraction #6. Second subculture cells were used.

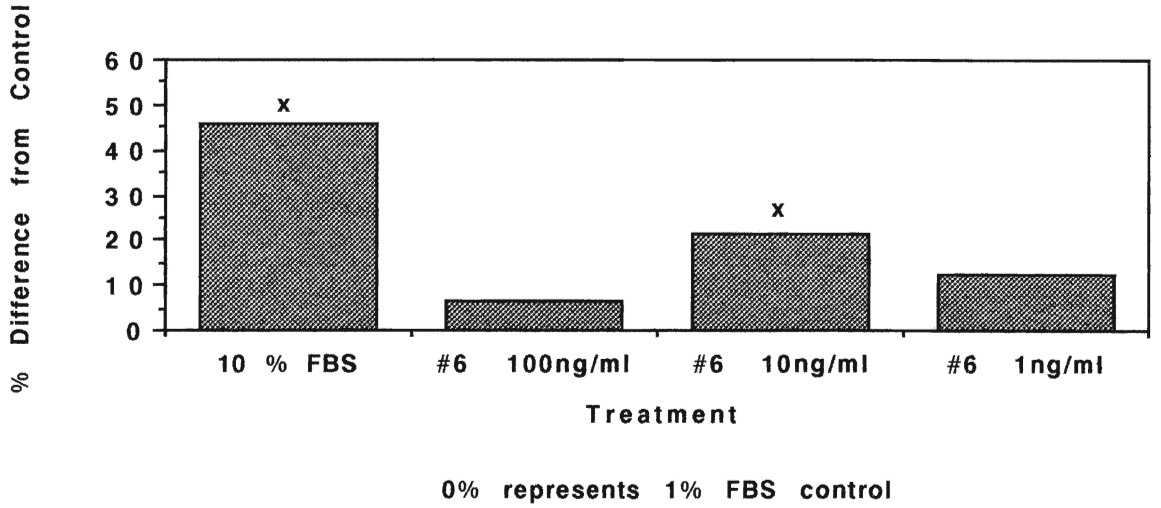
A


% Difference from Control
120
100
80
60
40
20
0
-20
-40
x
x
10 % FBS
#4 100ng/ml
#4 10ng/ml
#4 1ng/ml
Treatment
0% represents 1% FBS control

B


% Difference from Control
80
60
40
20
0
-20
-40
-60
x
x
10 % FBS
#5 100ng/ml
#5 10ng/ml
#5 1ng/ml
Treatment
0% represents 1% FBS control

C


% Difference from Control
60
50
40
30
20
10
0
x
x
10 % FBS
#6 100ng/ml
#6 10ng/ml
#6 1ng/ml
Treatment
0% represents 1% FBS control

10 ng/ml and 1 ng/ml. A significant 55.4% increase in label uptake in these cells was caused by 10% FBS.

As evidenced in Figure 15c, rp-HPLC fraction #6 promoted ^{3}H-thymidine incorporation in the non-neuronal cells; when tested at 10 ng/ml, a significant 21.3% increase was noted. The response displayed a bell-shaped concentration dependence. The increases in cell growth seen at 100 ng/ml and 1 ng/ml were not statistically significant. Supplying the cells with 10% FBS yielded a significant 45.5% increase in cell proliferation.

When rp-HPLC Fraction #7 was tested on the non-neuronal cells at 100 ng/ml, it caused a statistically significant 29.1% inhibition of ^{3}H-thymidine incorporation (Figure 16a). Interestingly, this same fraction at 10 ng/ml produced a significant stimulation in cell proliferation, with an observed 56.4% increase. This stimulation of growth over and above the 1% FBS control level was as high as that observed for 10% FBS (60.6%) in this assay. In fact, a Wilcoxon Mann-Whitney U test applied to this data indicated that there was no statistically significant difference between the mitogenic effects of rp-HPLC fraction #7 at 10 ng/ml and 10% FBS. At 1 ng/ml, rp-HPLC fraction #7 had no effect on cell growth. As can be seen, the response of the cells to rp-HPLC fraction #7 was dose-dependent, with a bell-shaped concentration dependence.

Although treatment with rp-HPLC fraction #8 at 100 ng/ml registered a 55.1% inhibition of ^{3}H-thymidine uptake and 10 ng/ml and 1 ng/ml of this material caused 24.1% and 10.4% increases respectively, none of these results were significantly different from the 1% FBS control (Figure 16b). This may have, in part, been due to a large variation in the cpm values obtained for ^{3}H-thymidine incorporation in the 1% FBS control of this particular assay. On the other hand, 10% FBS affected a significant 54.9% stimulation of cell growth.

Rp-HPLC fraction #9 was slightly biologically active at 100 ng/ml, causing a significant 4.8% increase in ^{3}H-thymidine uptake (Figure 16c).

Figure 16. Effect of rp-HPLC Fractions #7, 8, and 9 on ^{3}H-thymidine Incorporation in Cultured Chick Embryo Brain Non-neuronal Cells.

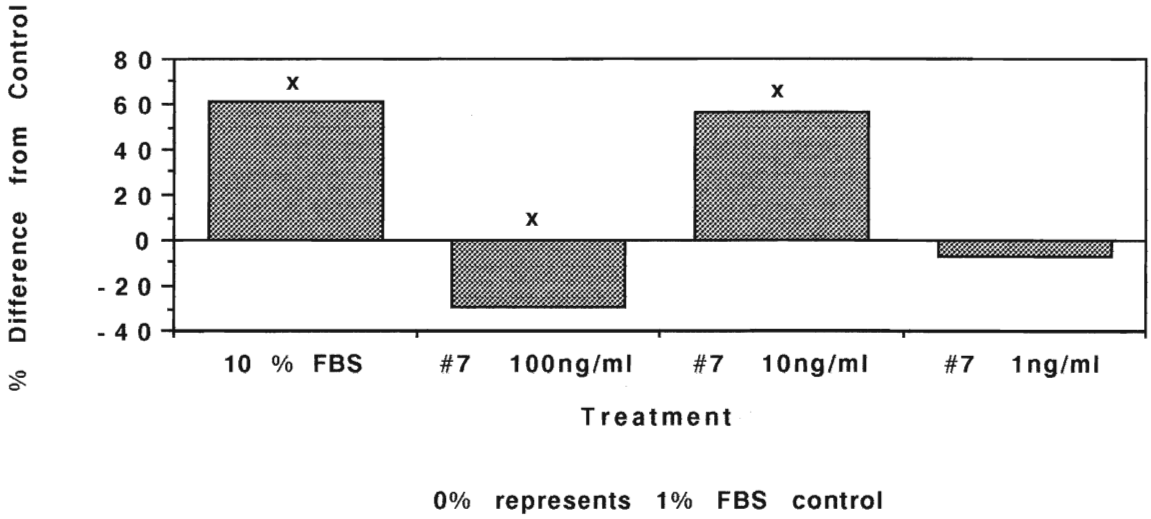
Data are presented as percent difference from the 1% FBS control for each of the rp-HPLC fraction concentrations tested and the 10% FBS control. Statistically significant differences ($p \leq 0.05$) are represented by an "x" over the corresponding bar on the graph. n = 5 for each rp-HPLC fraction test concentration and the 1% FBS control; n = 4 for the 10% FBS control. (rp-HPLC = reversed-phase high-performance liquid chromatography; FBS = fetal bovine serum)

A: Results for rp-HPLC fraction #7. Second subculture cells were used.

B: Results for rp-HPLC fraction #8. Second subculture cells were used.

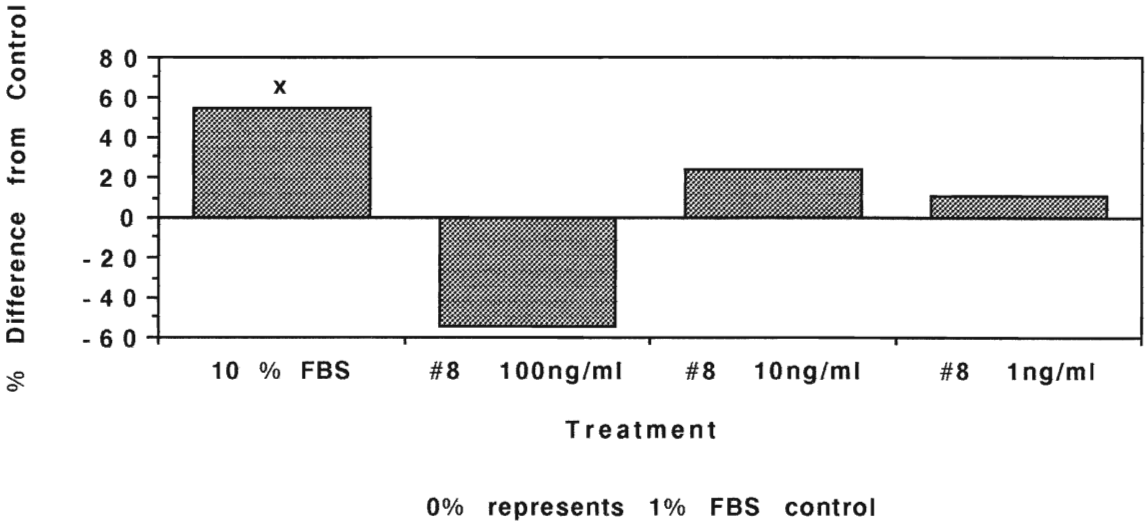
C: Results for rp-HPLC fraction #9. Third subculture cells were used.

A



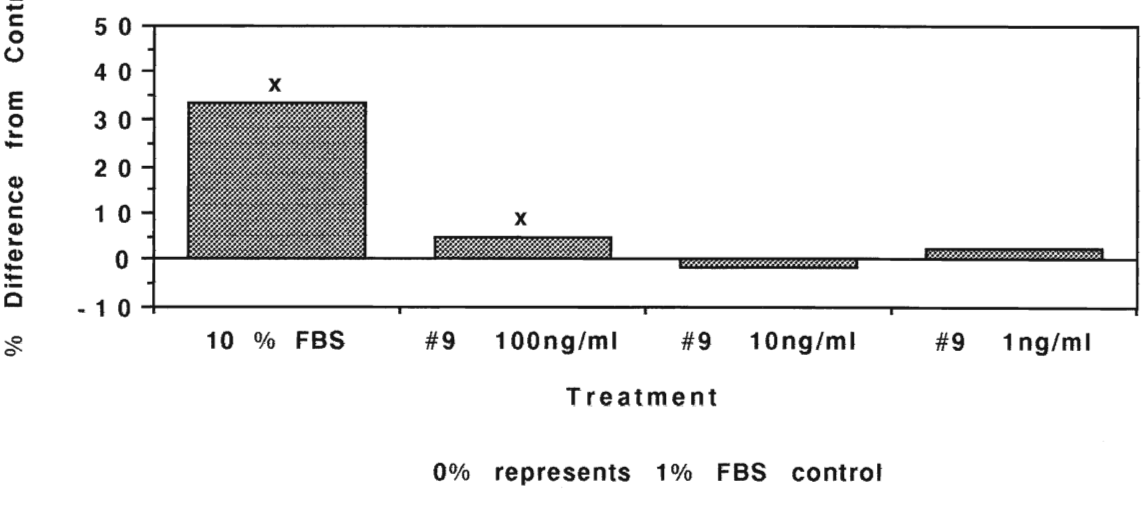
0% represents 1% FBS control

B



0% represents 1% FBS control

C



0% represents 1% FBS control

When tested at 10 ng/ml and 1 ng/ml, however, this fraction produced no apparent effect. A significant stimulation (32.9%) of non-neuronal cell growth occurred upon treatment with 10% FBS.

The bioassay results represented in Figure 17a indicate that rp-HPLC #10 had no effect on ^{3}H-thymidine incorporation at any of the tested concentrations. However, since exposure to 10% FBS also failed to significantly stimulate growth, the ability of the cells of this particular assay to respond to a growth stimulus is in question. Although the findings of this assay may be inconclusive, the trends observed in Figure 17a may still be valid.

Another fraction displaying some biological activity was rp-HPLC fraction #11. As evidenced in Figure 17b, at 100 ng/ml this material was ineffective. However, it was significantly mitogenic at 10 ng/ml and at 1 ng/ml demonstrated its greatest ability to promote incorporation of ^{3}H-thymidine with a significant 16.8% increase. The 10% FBS control caused a significant 61.1% stimulation of cell growth.

In summary, three of the rp-HPLC fractions were inhibitory to ^{3}H-thymidine uptake when tested at 100 ng/ml: rp-HPLC #4, #5, and #7. In contrast, five rp-HPLC fractions were biologically active causing increases in ^{3}H-thymidine incorporation in the non-neuronal cells: rp-HPLC #9 at 100 ng/ml, rp-HPLC #1, #6, #7, and #11 at 10 ng/ml, and rp-HPLC #11 at 1 ng/ml. Of these active fractions, rp-HPLC fraction #7 displayed the greatest mitogenicity, being as stimulatory as 10% FBS. Yet, rp-HPLC fraction #7 was also inhibitory at a higher concentration, indicating that it has a very narrow concentration dependence. Figure 18 presents a summary of the results for each rp-HPLC fraction tested at 10 ng/ml.

A final purification flow-chart showing each isolation step and the biologically active fraction obtained is presented in Figure 19. The protocol presented in this figure was found to be the most successful purification procedure.

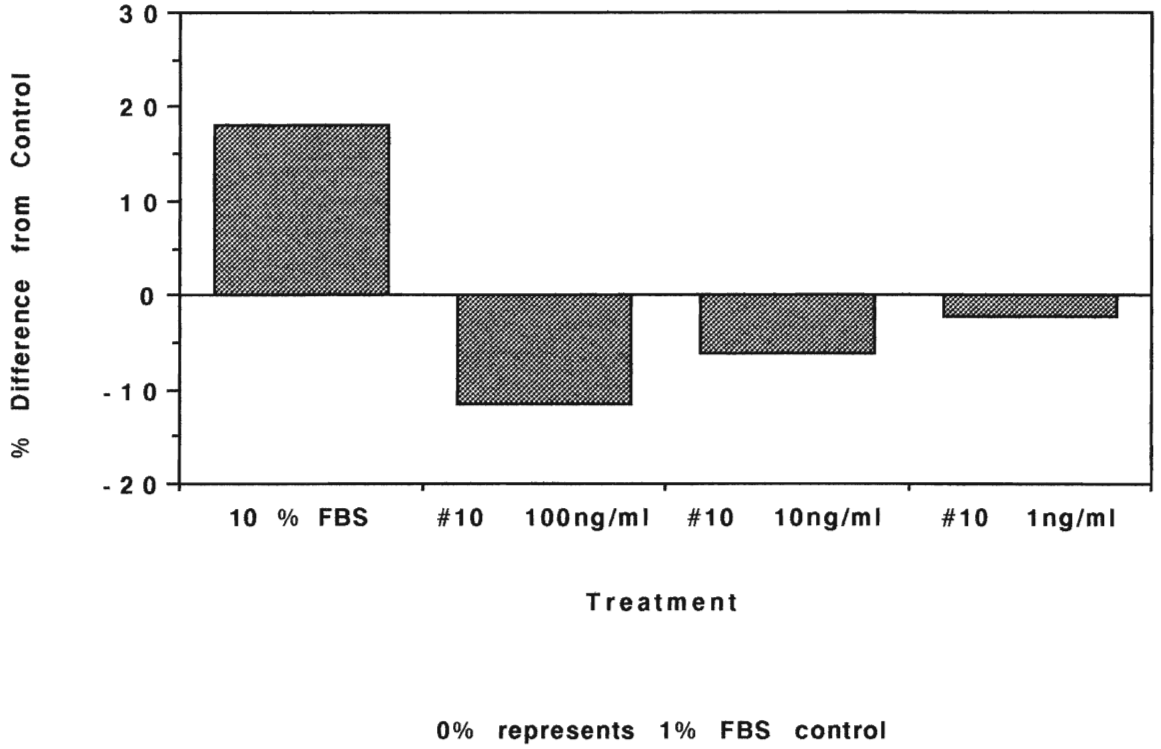
Figure 17. Effect of rp-HPLC Fractions #10 and 11 on ^{3}H-thymidine Incorporation in Cultured Chick Embryo Brain Non-neuronal Cells.

Data are presented as percent difference from the 1% FBS control for each of the rp-HPLC fraction concentrations tested and the 10% FBS control. Statistically significant differences ($p \leq 0.05$) are represented by an "x" over the corresponding bar on the graph. n = 5 for each rp-HPLC fraction test concentration and the 1% FBS control; n = 4 for the 10% FBS control. (rp-HPLC = reversed-phase high-performance liquid chromatography; FBS = fetal bovine serum)

A: Results for rp-HPLC fraction #10. Third subculture cells were used.

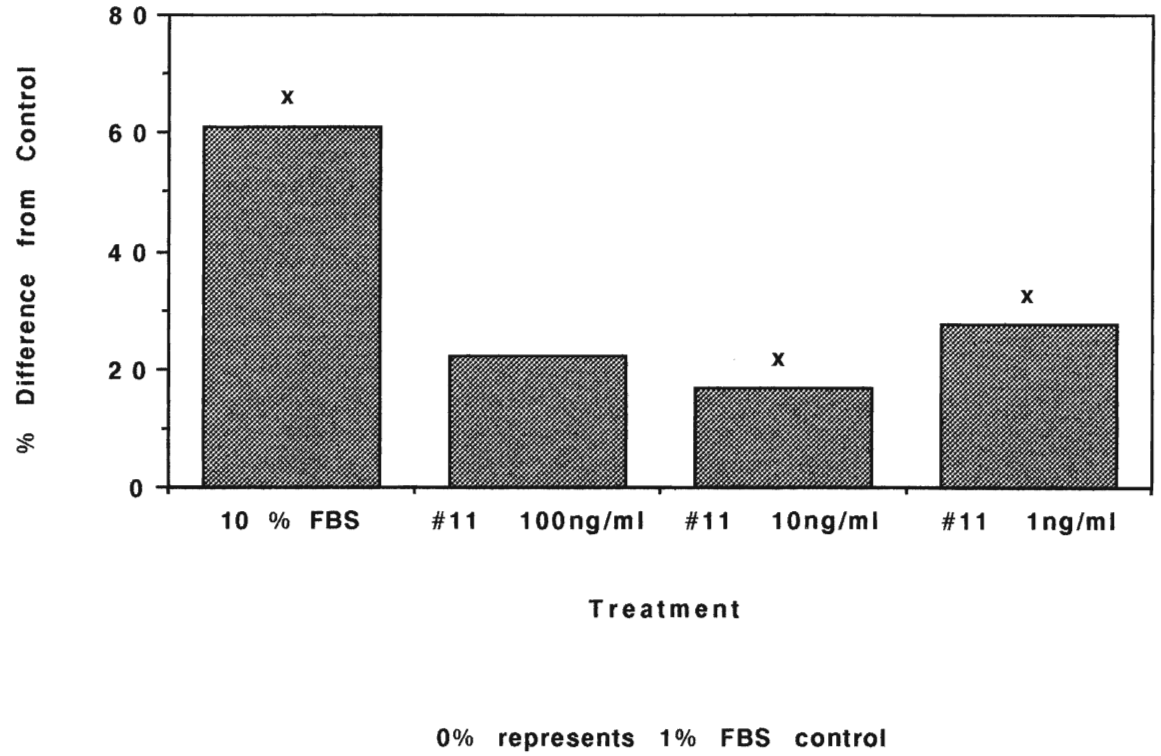
B: Results for rp-HPLC fraction #11. Third subculture cells were used.

A



0% represents 1% FBS control

B



0% represents 1% FBS control

Figure 18. Summary of the Effects of rp-HPLC Fractions #1-11 on ^{3}H-thymidine Incorporation in Cultured Chick Embryo Brain Non-neuronal Cells.

The graph represents a compilation of data from individual bioassays for each rp-HPLC fraction tested at 10 ng/ml. Data are presented as percent difference from the corresponding 1% FBS control for each of the rp-HPLC fractions. Statistically significant differences ($p \leq 0.05$) are represented by an "x" over the corresponding bar on the graph. n = 5 for each rp-HPLC fraction and the 1% FBS control. (rp-HPLC = reversed-phase high-performance liquid chromatography; FBS = fetal bovine serum)

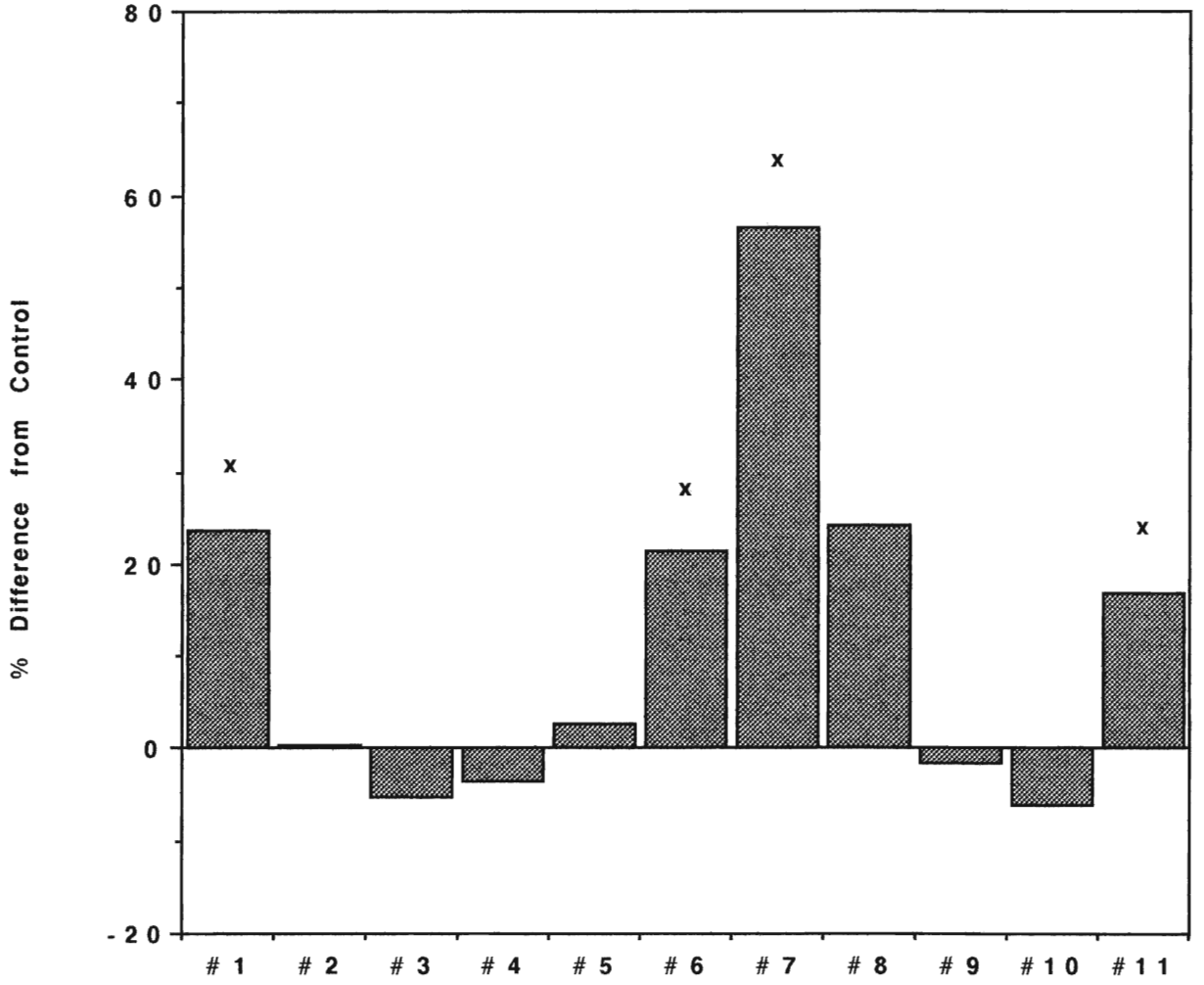
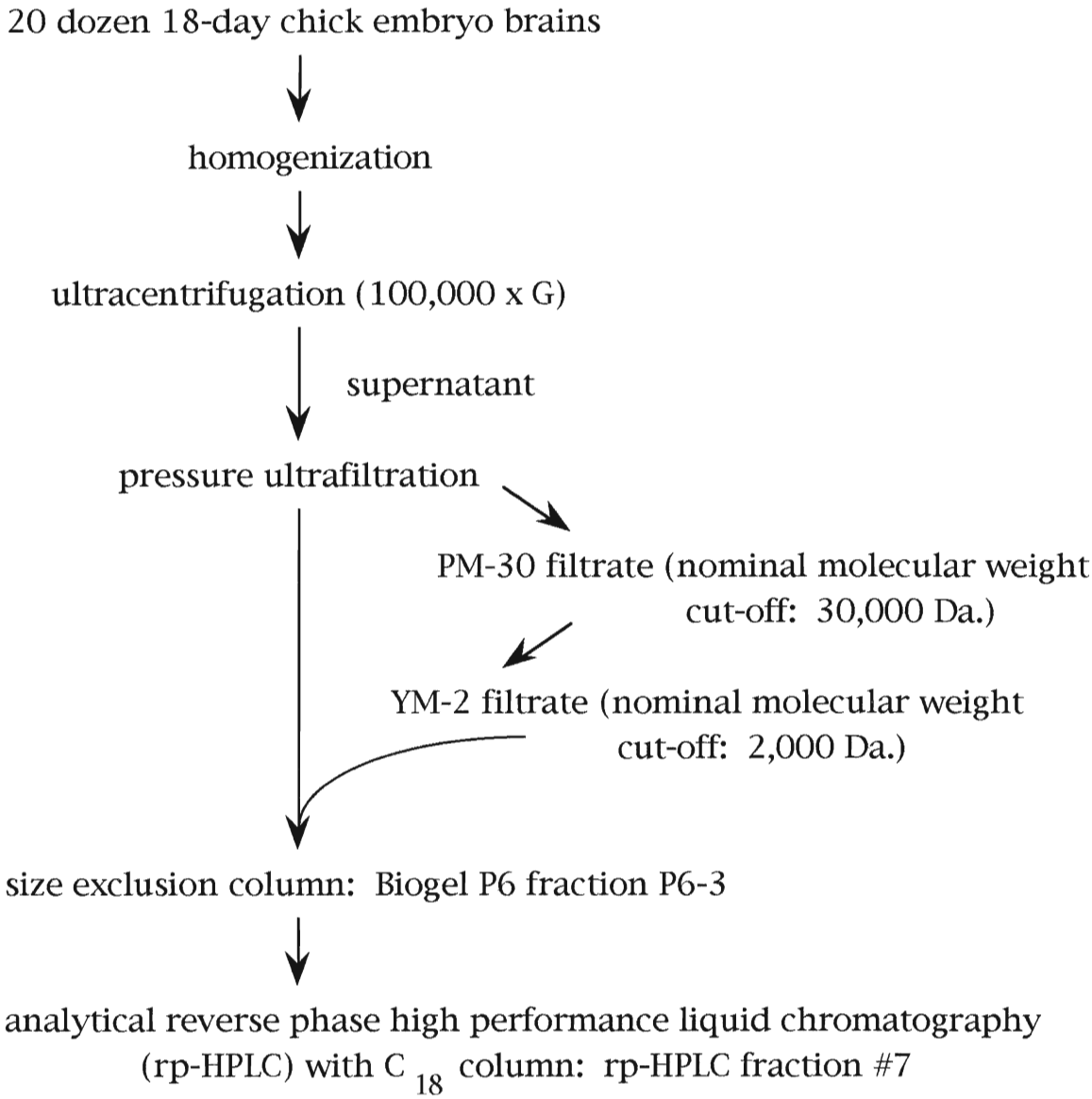

80
60
40
20
0
-20
% Difference from Control
x
x
x
x
1
2
3
4
5
6
7
8
9
10
11
rpHPLC Fraction Number (10 ng/ml)
0% represents 1% FBS control

Figure 19. Optimal CBGF Purification Procedure Showing the Biologically Active Fraction Obtained at Each Step.


20 dozen 18-day chick embryo brains
homogenization
ultracentrifugation (100,000 x G)
supernatant
pressure ultrafiltration
PM-30 filtrate (nominal molecular weight cut-off: 30,000 Da.)
YM-2 filtrate (nominal molecular weight cut-off: 2,000 Da.)
size exclusion column: Biogel P6 fraction P6-3
analytical reverse phase high performance liquid chromatography (rp-HPLC) with C_{18} column: rp-HPLC fraction #7

F. Specific activity calculations

A summary of the degree of purification that was achieved is given in Table III. Initially, 1,155 mg of protein were isolated from the 100,000xG supernatant isolated from 20 dozen chick embryo brains. Following ultrafiltration through the PM-30 membrane, 14.2 mg of protein remained in the filtrate. This material had a half-maximal stimulation of ^{3}H-thymidine incorporation (ED_{50}) in the non-neuronal cells of 5000 ng/ml. The mitogen was further purified by passing it through a YM-2 membrane; the resultant amount of protein in the filtrate was 11.6 mg. The YM-2 filtrate had an ED_{50} of 540 ng/ml. This represented a 9.26 fold purification over the PM-30 filtrate. After eluting the active material from the P6 column, only 0.42 mg of protein remained. Fraction P6-3 had an ED_{50} of 18 ng/ml and was 278 times purer than the PM-30 filtrate. Following the final isolation step, rp-HPLC fraction #7 contained 0.02 mg of protein and had an ED_{50} of 5.3 ng/ml. Thus, this corresponded to a final CBGF purification factor of 943 when compared to the PM-30 filtrate.

Characterization of Chick Brain Growth Factor's Biochemical Properties

The third goal of this research was to gain insight into some of the biochemical characteristics of the biologically active fraction. Using the purification procedure previously reported (Carlone *et al.*, 1987), a low molecular weight (approximately 1,500 Da.) fifteen amino acid peptide was isolated. Other researchers investigating this problem have used this method but were successful in isolating an active fraction using the UM-O5 replacement membrane, YC-05 (Kim *et al.*, 1991). Employment of this different technique has resulted in the purification of substances identified as guanosine 5'-monophosphate (GMP) (Kim *et al.*, 1991) and adenosine

Table III. Summary of the Purification of CBGF from Chick Embryo Brain

Purification step	Total Protein (mg)	ED_{50} [a] (ng/ml)	Purification factor
100,000 x G supernatant	1155	-	-
PM-30 filtrate	14.2	5000	-
YM-2 filtrate	11.6	540	9.26
Fraction P6-3	0.42	18	278
rp-HPLC fraction #7	0.02	5.3	943

The biological activity was purified from 20 dozen chick embryo brains as described in the text .

[a] ED_{50} is the quantity of a given fraction necessary to cause half-maximal stimulation of ^{3}H-thymidine incorporation in the non-neuronal cell bioassay outlined in the text.

The purification factor was calculated by dividing the ED_{50} of the PM-30 filtrate by the ED_{50} of the desired active fraction.

5'-monophosphate (AMP) (Rathbone *et al.*, 1992a) both of which were found to be biologically active on chick embryo brain astrocytes. The current research project, presented in this thesis, has also changed the purification protocol. A variety of methods were used to attempt a partial characterization of the mitogenic substance.

A. Ultraviolet absorbance spectra of CBGF, GMP, and AMP

The ultraviolet (UV) spectrum of rp-HPLC fraction #7 reconstituted in distilled H_2O was taken; the spectrum from 200 nm to 300 nm is shown in Figure 20. A symmetrical absorbance peak was observed at 257.5 nm. A second peak was evident at 203 nm. A peptide sample having tyrosine and tryptophan residues would be expected to absorb UV light at 280 nm (Scopes, 1982). Samples containing nucleic acids, on the other hand, absorb UV light at 256 nm. The peak at 257.5 nm in the absorbance spectrum of rp-HPLC fraction #7 indicated that a nucleoside or nucleotide may have been present in the sample. Moreover, other researchers have isolated both GMP and AMP using a variation of the purification procedure employed in this work (Kim *et al.*, 1991; Rathbone *et al.*, 1992a) For these reasons, the UV spectra of both GMP and AMP were also determined.

5'-GMP was dissolved in distilled water and its UV spectrum between 200 nm and 300 nm was recorded (Figure 21). An asymmetrical peak having a high wavelength shoulder and an absorbance peak maximum at 252 nm was observed.

When the UV spectrum of 5'-AMP in distilled water was taken, a symmetrical absorbance peak at 257.5 nm was found; a second peak appeared at 208.5 nm (Figure 22).

In addition, UV spectra of the various buffer components and solvents used in the purification of the biologically active material were determined. Ammonium acetate, K_2HPO_4, and KH_2PO_4 were individually

Figure 20. Ultraviolet Absorbance Spectrum of rp-HPLC Fraction #7.

The lyophilized sample was reconstituted in distilled H_2O. The spectrum was scanned from 200 nm to 300 nm.

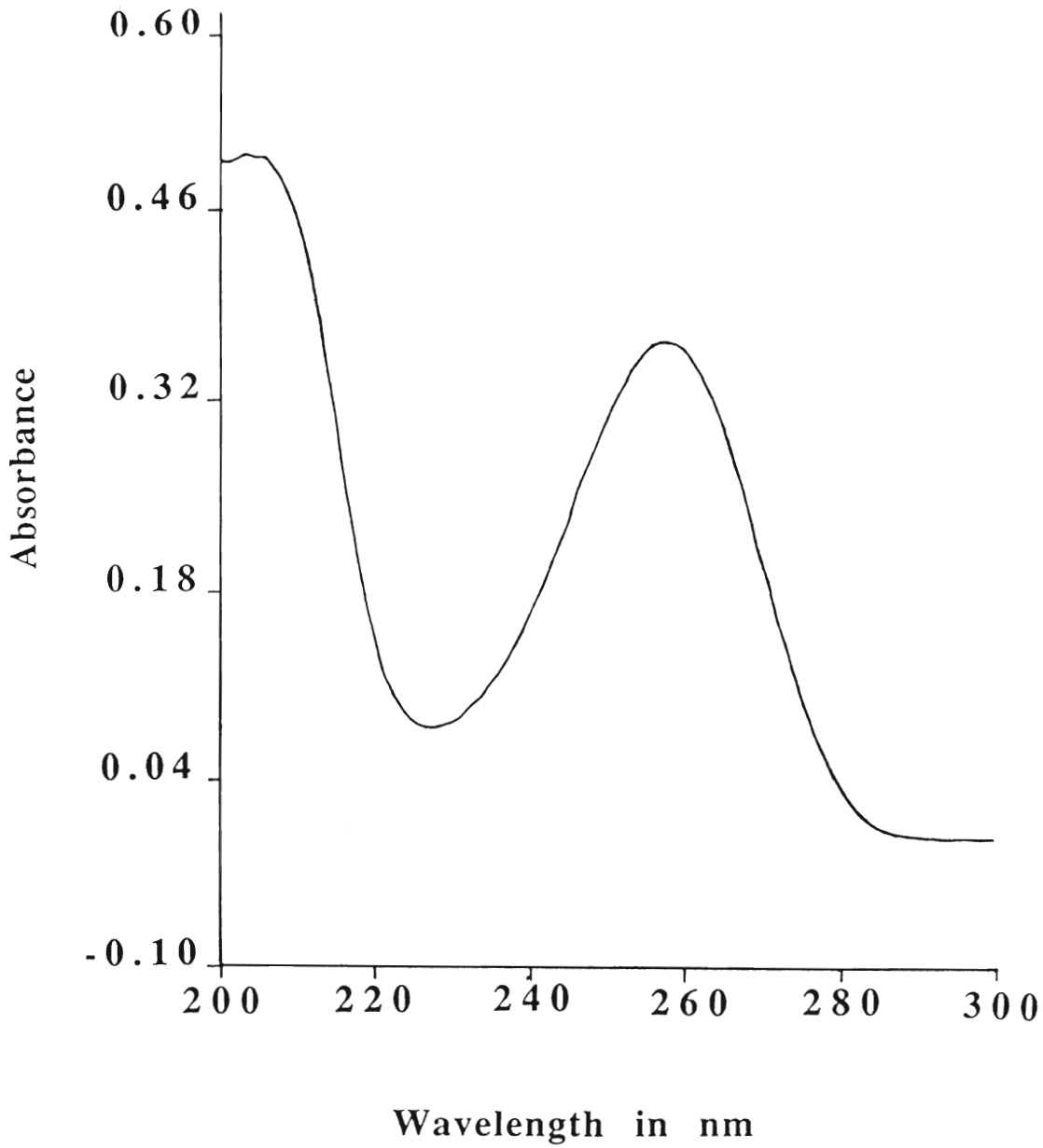

0.60
0.46
0.32
0.18
0.04
-0.10
Absorbance
200
220
240
260
280
300
Wavelength in nm

Figure 21. Ultraviolet Absorbance Spectrum of 5'-GMP.

The sample was dissolved in distilled H_2O. The spectrum was scanned from 200 nm to 300 nm.
(5'-GMP = guanosine 5'-monophosphate)

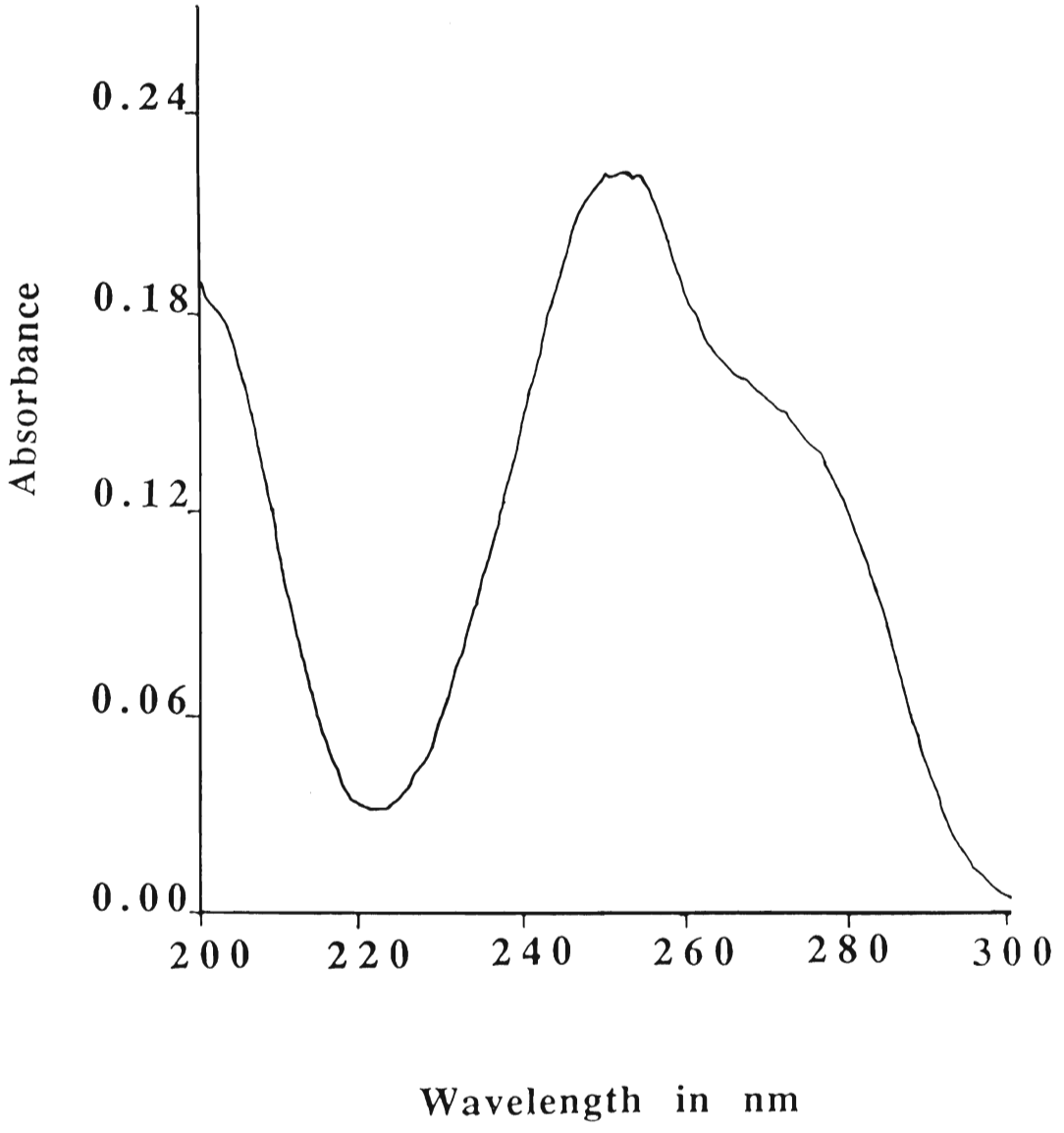
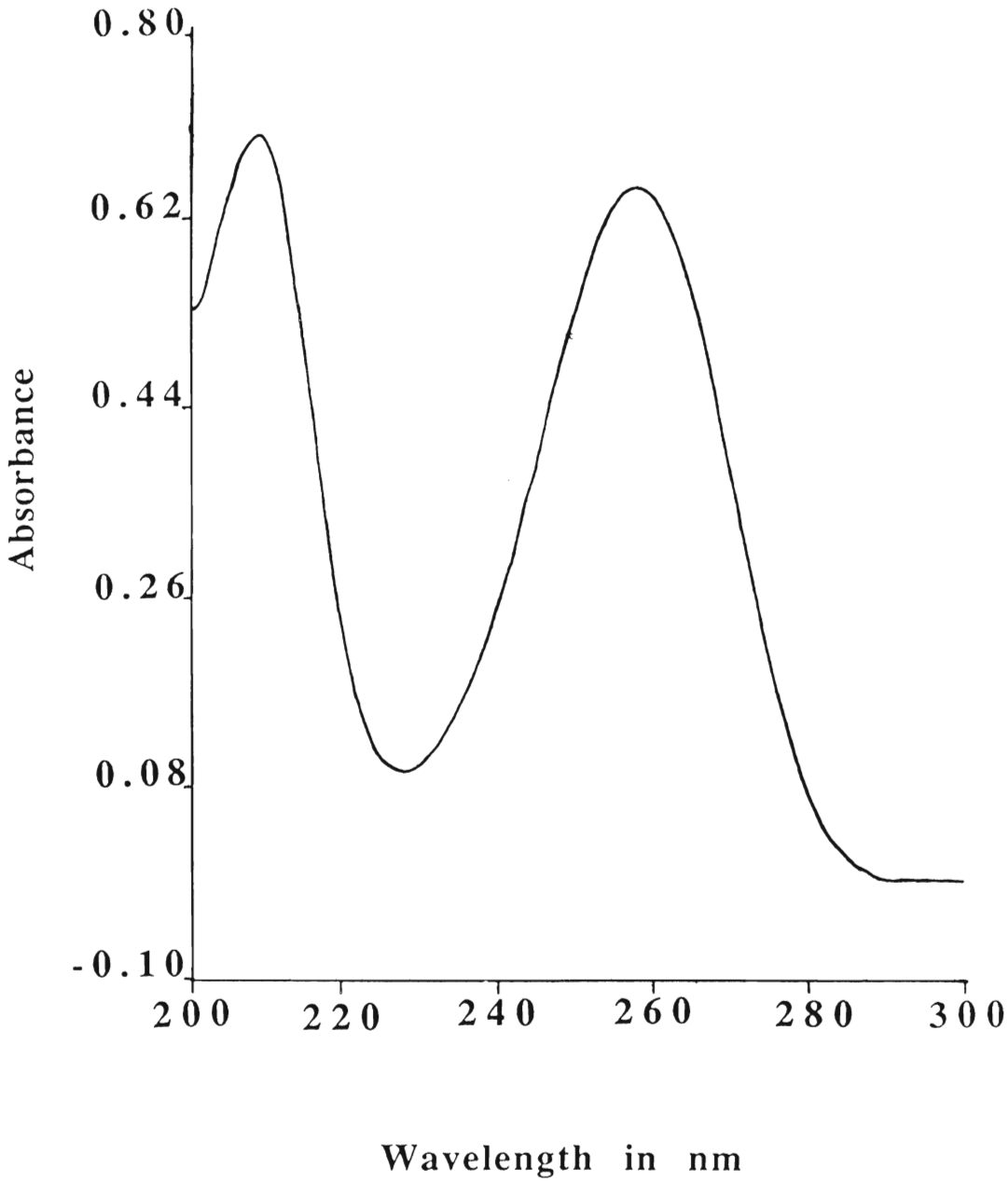

0.24
0.18
0.12
0.06
0.00
Absorbance
200
220
240
260
280
300
Wavelength in nm

Figure 22. Ultraviolet Absorbance Spectrum of 5'-AMP.

The sample was dissolved in distilled H_2O. The spectrum was scanned from 200 nm to 300 nm.
(5'-AMP = adenosine 5'-monophosphate)


0.80
0.62
0.44
0.26
0.08
-0.10
Absorbance
200
220
240
260
280
300
Wavelength in nm

dissolved in distilled H_2O and the UV spectrum of each was scanned between 200 nm and 300 nm. Ammonium acetate displayed an absorbance maximum at 230 nm. K_2HPO_4 had absorbance maxima at 205 nm and 201 nm. KH_2PO_4 absorbed increasing amounts of UV light as the wavelength approached 200 nm; no absorbance peak was observed within the scan limits. Neat HPLC grade acetonitrile had a small absorbance peak at 214 nm. Neither the buffer salts nor the HPLC solvent absorbed at 257.5 nm, the wavelength at which rp-HPLC fraction #7 had an absorbance peak.

B. Comparison of GMP and AMP reverse phase HPLC elution profiles

In light of the identification of a biologically active fraction from the 18-day chick embryo as GMP (Kim *et al.*, 1991), a comparison was made between the reverse phase HPLC elution profiles of rp-HPLC fraction #7 and 5'-GMP. Kim and his colleagues had found that their mitogenic fraction and 5'-GMP co-eluted when tested under the same conditions.

In this current research project, fraction P6-3, the biologically active material isolated from the Biogel P6 column elution, was applied to a C_{18} reverse phase HPLC column for further purification. A gradient of 0.1% to 99.9 % CH_3CN in H_2O with 0.05% TFA in 20 minutes was employed to elute eleven fractions, of which rp-HPLC fraction #7 was the most mitogenic. A typical elution profile recorded at 280 nm is shown in Figure 12. Rp-HPLC fraction #7 eluted as a fairly sharp and symmetrical peak. From ten replicate elution profiles, an average time for rp-HPLC fraction #7 elution was calculated. The mean (+/- standard deviation) onset of elution of peak #7 was 7.1 +/- 0.3 minutes; this corresponded to 35.5 +/- 1.5% CH_3CN. At its point of maximum absorbance, rp-HPLC fraction #7 eluted at 39.2 +/- 1.9% CH_3CN.

In contrast, 5'-GMP had a much different elution profile (Figure 23a recorded at 280 nm) when eluted under identical conditions as those employed in the isolation of rp-HPLC fraction #7. A broad peak which began eluting at 11.8 minutes or 59.0% CH_3CN was apparent. This peak had a shoulder which eluted at 75.9% CH_3CN.

Since other work by Rathbone and his co-workers (Rathbone *et al.*, 1992a) had revealed that AMP could be isolated from the 18 day chick embryo brain and that it was a mitogen for chick brain non-neuronal cells, 5'-AMP was also eluted under identical conditions as those utilized to separate rp-HPLC fraction #7. A typical elution profile of 5'-AMP is shown in Figure 23b. A sharp symmetrical peak initiated elution at 11.0 minutes or 55.0% CH_3CN. Like 5'-GMP, 5'-AMP eluted much later than rp-HPLC fraction #7.

C. Proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) of rp-HPLC fraction #7

In order to gain some information regarding the molecular structure of the material comprising rp-HPLC fraction #7, the lyophilized sample was reconstituted in D_2O and a ^{1}H-NMR spectrum was run. Unfortunately, no signal was seen due to the inadequate sample size. Approximately 1 mg of pure sample is required to detect a ^{1}H-NMR signal. In large molecules such as peptides and proteins, a larger sample is necessary since the molarity of individual protons is low.

D. Biological activity of the nucleotides GMP, cGMP, AMP, and cAMP

The purification employed by Kim *et al.* (1991) resulted in the identification of a mitogenic fraction from chick embryo brains as GMP;

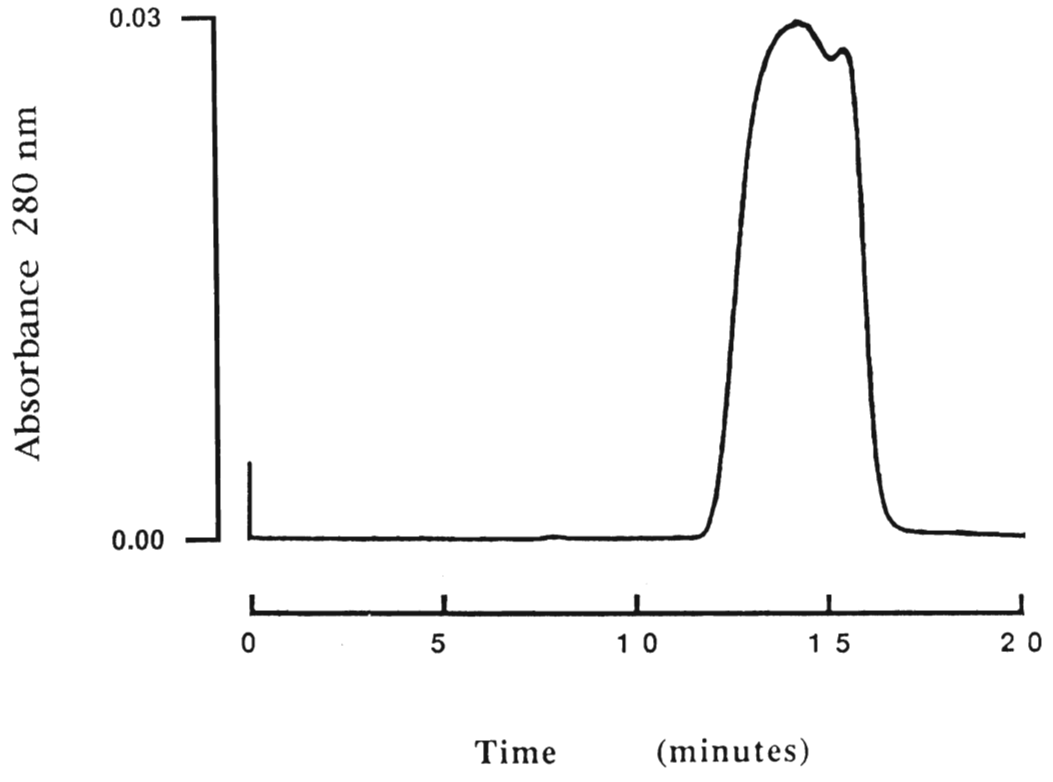
Figure 23. RP-HPLC Elution Profiles of 5'-GMP and 5'-AMP.

A μBondapak C_{18} column was used to individually chromatograph commercially available 5'-GMP and 5'-AMP under identical conditions as those employed in the isolation of rp-HPLC fraction #7, namely a gradient of 0.1% to 99.9% CH_3CN in H_2O with 0.05% TFA in 20 minutes. The elution profiles were monitored at 280 nm. (5'-GMP = guanosine 5'-monophosphate; 5'-AMP = adenosine 5'-monophosphate; RP-HPLC = reversed phase high-performance liquid chromatography; TFA = trifluoroacetic acid)

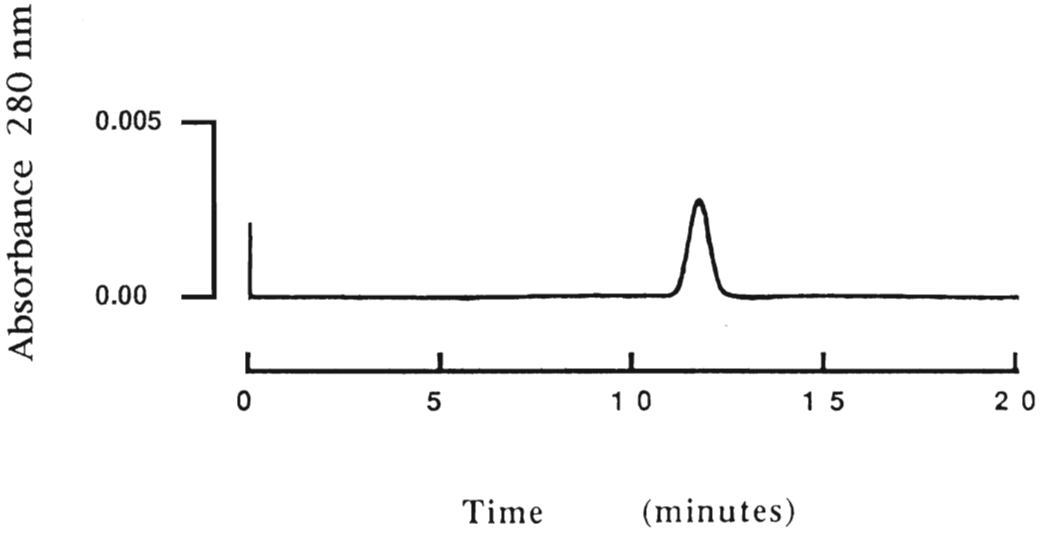
A: Rp-hplc elution profile of 5'-GMP.

B: Rp-hplc elution profile of 5'-AMP.

A


0.03
0.00
Absorbance 280 nm
0
5
10
15
20
Time (minutes)

B


0.005
0.00
Absorbance 280 nm
0
5
10
15
20
Time (minutes)

the isolated material was biologically active on the chick brain non-neuronal cells at 50 μM. These researchers used the same protocol to isolate AMP from the chick embryo brain; it maximally stimulated chick astrocyte growth at 500 nM (Rathbone *et al.*, 1992a). The research project outlined in this thesis endeavoured to determine the biological activity of commercially available 5'-GMP and 5'-AMP, as well as a number of other nucleotides. The chick embryo brain non-neuronal cell bioassay was used as it had been throughout this work.

Commercially available 5'-GMP was tested for its biological activity on the non-neuronal cells. Two 24 well plates were assayed, each with three concentrations of GMP; the results are depicted in Figure 24a and b. From Figure 24a, it is apparent that at 50 μM, GMP was inhibitory to cell proliferation and caused a significant 29.0% decrease in ^{3}H-thymidine incorporation. The inhibition of cell growth seen at GMP concentrations of 500 μM and 5 μM were not statistically significant. The control 10% FBS was significantly stimulatory affecting a 94.2% increase in cell growth. The results of Figure 24b indicate that low concentrations of GMP were also inhibitory to ^{3}H-thymidine incorporation in the test cells. At 0.5 μM, GMP produced a significant 24.8% decline in non-neuronal cell growth when compared to treatment with the 1% FBS control. A concentration of 0.05 μM GMP also caused a significant decrease in cell proliferation of 23.1%. Similarly, 0.005 μM GMP significantly inhibited cell growth by 14.7%. Cell proliferation was significantly increased 58.1% by 10% FBS in this assay.

Cyclic GMP (cGMP) was also tested in the non-neuronal bioassay (Figure 25). At the highest concentration examined, 5450 nM cGMP was inhibitory to cell growth and caused a significant 46.1% decrease in ^{3}H-thymidine incorporation in the test cells. No effect on the cells was registered when cGMP was assayed at 545 nM. However, at 5.45 nm, cGMP was most biologically active and affected a statistically significant

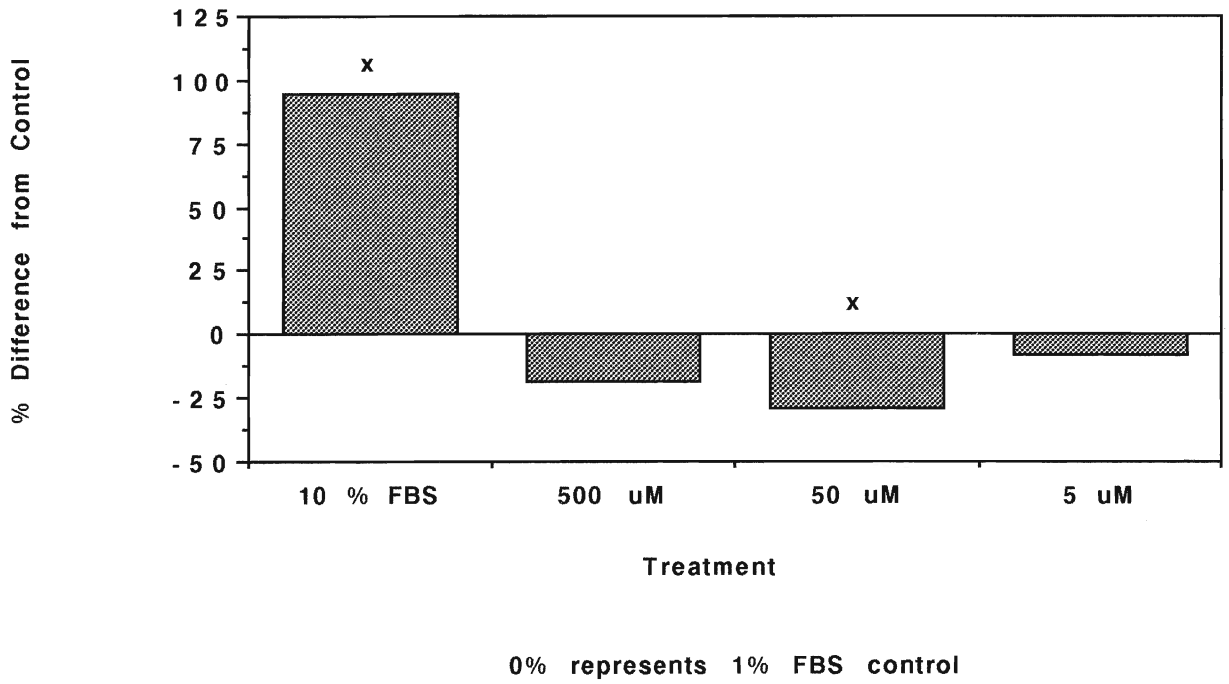
Figure 24. Effect of 5'-GMP on ^{3}H-thymidine Incorporation in Cultured Chick Embryo Brain Non-neuronal Cells.

Data are presented as percent difference from the 1% FBS control for each of the 5'-GMP concentrations tested and the 10% FBS control. Statistically significant differences ($p \leq 0.05$) are represented by an "x" over the corresponding bar on the graph. n = 5 for each 5'-GMP test concentration and the 1% FBS control; n = 4 for the 10% FBS control. Second subculture cells were used. (5'-GMP = guanosine 5'-monophosphate; FBS = fetal bovine serum)

A: 5'-GMP concentrations tested: 500 μM, 50 μM, and 5 μM.

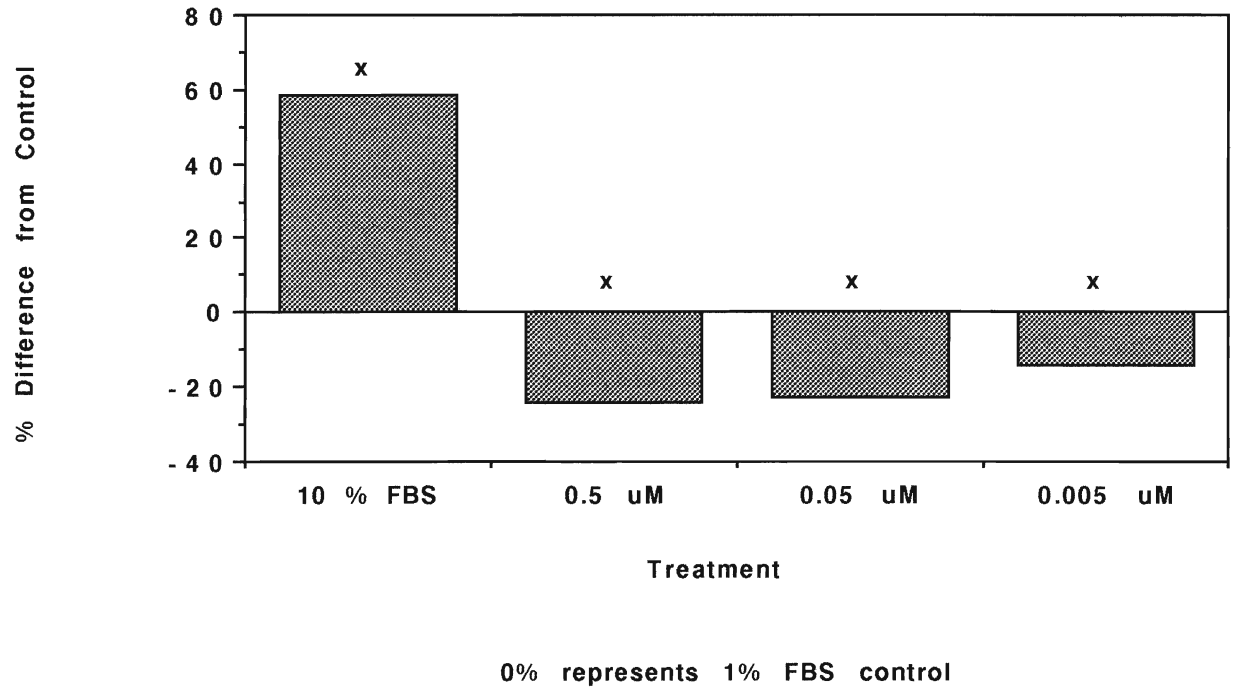
B: 5'-GMP concentrations tested: 0.5 μM, 0.05 μM, 0.005 μM.

A



0% represents 1% FBS control

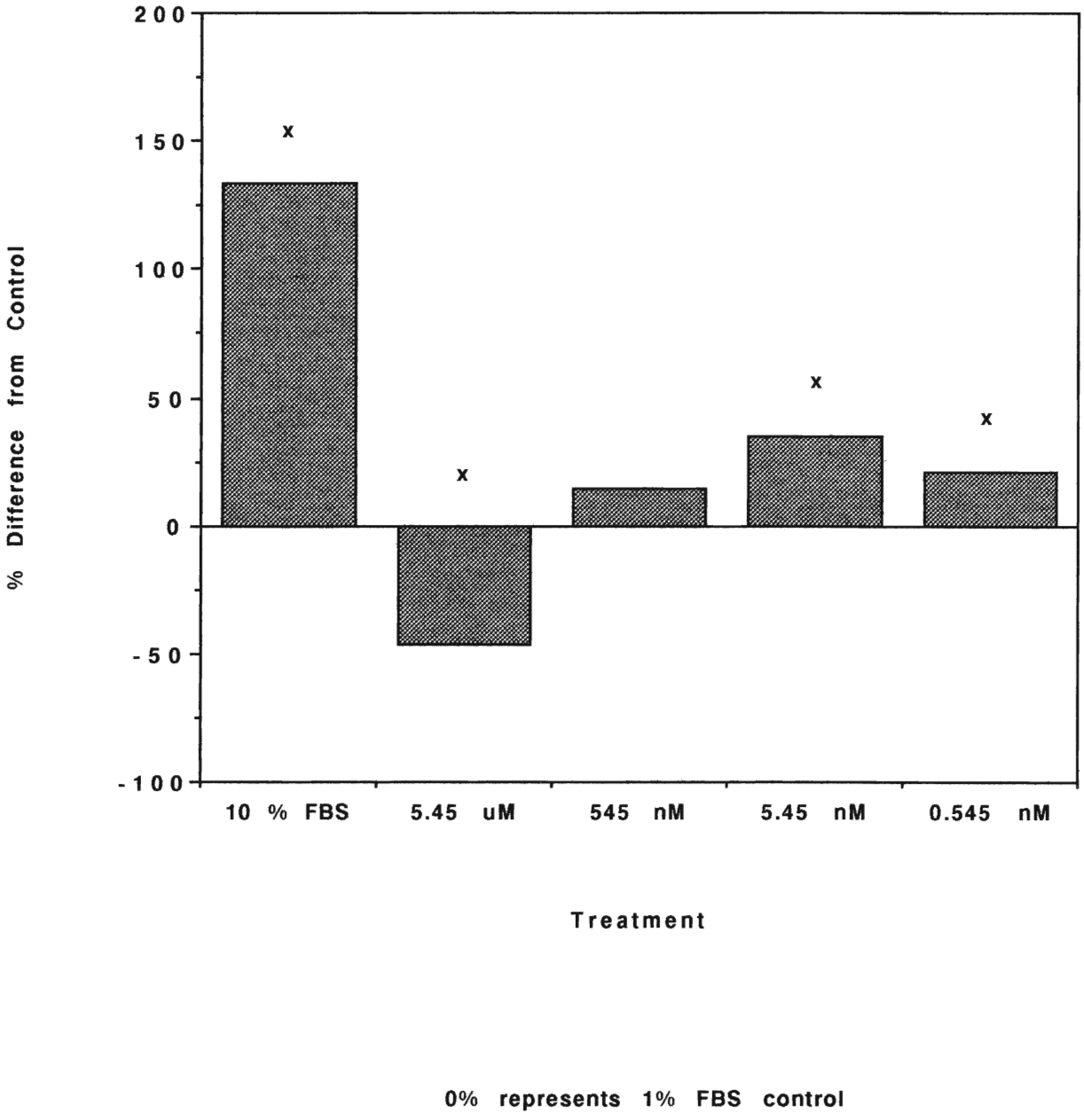
B



0% represents 1% FBS control

Figure 25. Effect of cGMP on ^{3}H-thymidine Incorporation in Cultured Chick Embryo Brain Non-neuronal Cells.

Data are presented as percent difference from the 1% FBS control for each of the cGMP concentrations tested and the 10% FBS control. Statistically significant differences ($p \leq 0.05$) are represented by an "x" over the corresponding bar on the graph. n = 4 for each cGMP test concentration and the 1% and 10% FBS controls. Second subculture cells were used. (cGMP = cyclic guanosine monophosphate; FBS = fetal bovine serum)



0% represents 1% FBS control

35.4% increase in ^{3}H-thymidine uptake. At 0.545 nm, this nucleotide also significantly stimulated a 21.1% increase in cell growth. As is evident, the biological effects of cGMP manifest themselves in a dose-dependent fashion. In this assay, treatment with 10% FBS resulted in a significant 133.6% rise is radio-label uptake.

When AMP was assayed, it was found to have no effect on non-neuronal cell growth (Figure 26a). It was tested at 50 μM, 5 μM, 0.5 μM, and 0.005 μM, but it caused no statistically significant change in ^{3}H-thymidine incorporation in the cells when compared to the 1% FBS control. However, 10% FBS produced a significant 209.8% stimulation of cell growth.

Treatment with cyclic AMP (cAMP) also caused no change to the ^{3}H-thymidine uptake of the non-neuronal cells. There was no statistically significant effect on cell growth when cAMP was tested at 50 μM, 5 μM, 0.5 μM, and 0.05 μM. On the other hand, 10% FBS affected a significant 127.8% increase in ^{3}H-thymidine incorporation in these cells. These results may be seen in Figure 26b.

E. Effect of pronase treatment on the biological activity of P6-3

Another method which was used to contribute information to the characterization of CBGF was pronase treatment of the biologically active material; the sample subjected to this non-specific proteolytic digestion was then tested for activity in the non-neuronal cell bioassay. With such a test, one would expect to observe a decrease in biological activity if the mitogen in question were of a peptide or protein nature. If the mitogen were some other type of molecule, one would anticipate that pronase treatment would have no effect on biological activity.

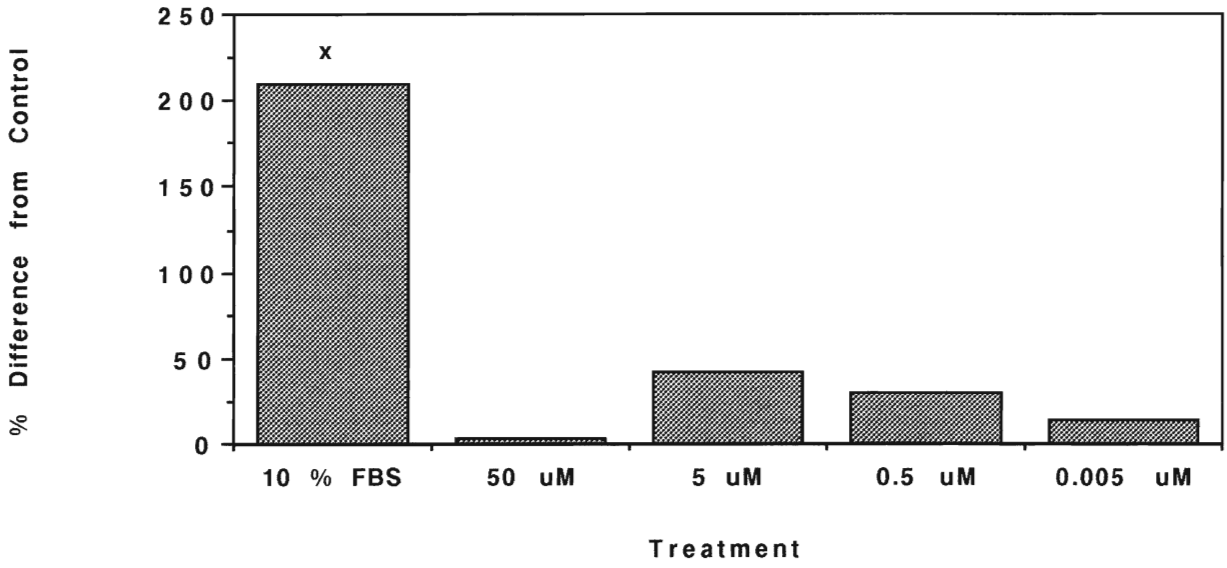
Figure 26. Effect of 5'-AMP and cAMP on ^{3}H-thymidine Incorporation in Cultured Chick Embryo Brain Non-neuronal Cells.

Data are presented as percent difference from the 1% FBS control for each of the nucleotide concentrations tested and the 10% FBS control. Statistically significant differences ($p \leq 0.05$) are represented by an "x" over the corresponding bar on the graph. n = 4 for each 5'-AMP and cAMP test concentration and the 1% and 10% FBS controls. (5'-AMP = adenosine 5'-monophosphate; cAMP = cyclic adenosine monophosphate; FBS = fetal bovine serum)

A: Results are given for 5'-AMP. Second subculture cells were used.

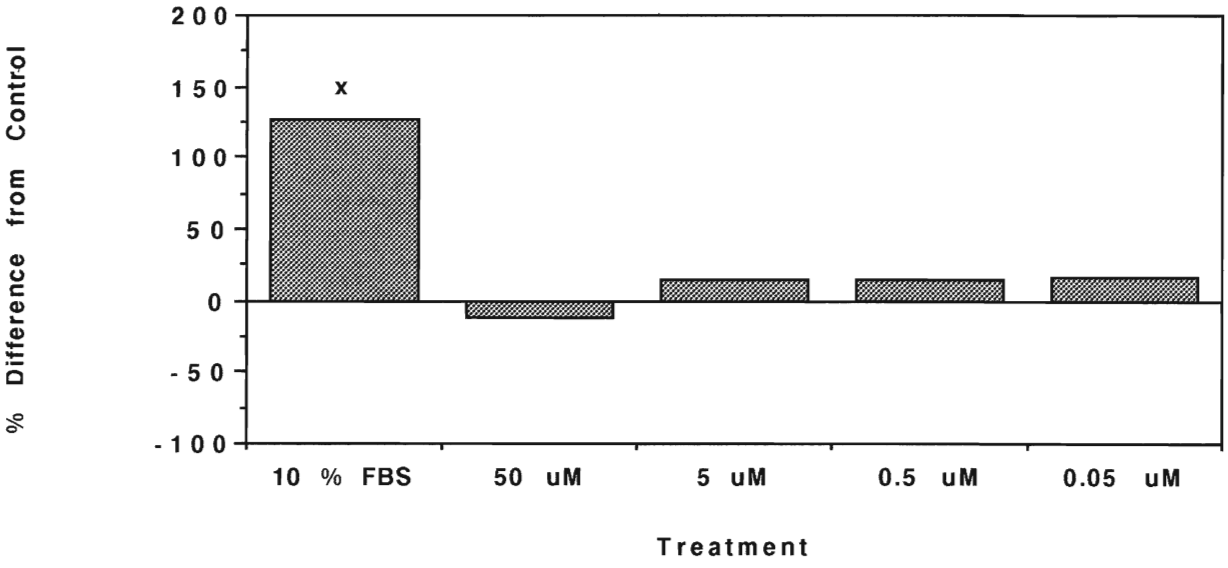
B: Results are given for cAMP. Third subculture cells were used.

A



0% represents 1% FBS control

B



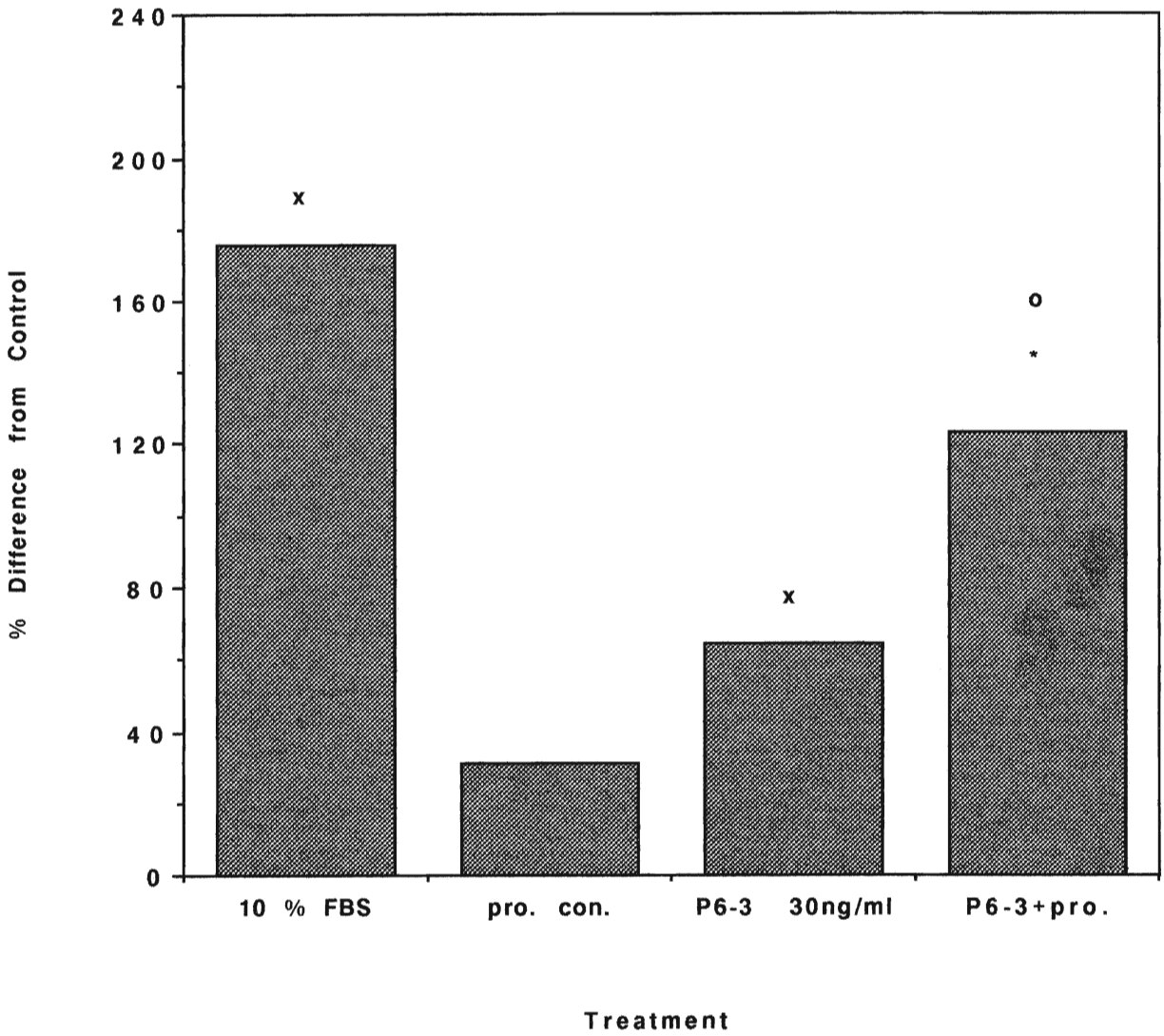
0% represents 1% FBS control

The active fraction P6-3 was incubated with pronase and the resultant mixture was used in the bioassay. In addition to 1% and 10% FBS, two other controls were included in this assay. The first was an incubation of distilled H_2O with pronase, employed in the test procedure to determine the effects of pronase alone on chick brain non-neuronal cell growth. The second control involved the treatment of cells with undigested P6-3, which would stimulate cell proliferation. In this way, it was possible to compare the effects of the pronase treated mitogen with the unaltered factor while taking into consideration the extraneous effects of the pronase itself. The results of this assay are presented in Figure 27. The pronase alone (pro. con.) had no statistically significant effect on cell growth. As previously noted, fraction P6-3 at 30 ng/ml caused a significant 64.3% increase in ^{3}H-thymidine incorporation in the non-neuronal cells. Surprisingly, P6-3 treated with pronase produced a statistically significant 123.0% stimulation of ^{3}H-thymidine uptake in the cells. Not only was this increase significantly greater than 1% FBS, but more importantly it represented stimulation over levels achieved with both pronase and the active fraction P6-3 alone. These findings were confirmed using the P6-3 fraction isolated from a different preparation. The amount of P6-3 used for pronase digestion corresponded to a final concentration of 30 ng/ml when administered to the non-neuronal cells. The 10% FBS control promoted a significant 175.7% increase in ^{3}H-thymidine uptake in the test cells.

In order to determine whether the stimulation of cell growth achieved with pronase treated P6-3 was due to an increase in the extracellular amino acid pool, a control assay was performed. Bovine serum albumin (BSA) was incubated with pronase under the conditions employed in the pronase treated P6-3 assay. A pronase control in which pronase and distilled H_2O were incubated together was also included to ascertain any effects the pronase might have on cell growth. Untreated

Figure 27. Effect of Pronase Treated P6-3 on ^{3}H-thymidine Incorporation in Cultured Chick Embryo Brain Non-neuronal Cells.

P6-3 was incubated with pronase and tested at a P6-3 concentration of 30 ng/ml (P6-3 + pro.); controls of distilled H_2O incubated with pronase (pro. con.) and untreated P6-3 at 30 ng/ml (P6-3 30 ng/ml) were also tested. Data are presented as percent difference from the 1% FBS control for each of the treatments tested and the 10% FBS control. n = 4 for the 1% and 10% FBS controls and the pronase control; n = 6 for the untreated P6-3 and the pronase treated P6-3. Second subculture cells were used. (FBS = fetal bovine serum)
Statistically significant differences ($p \leq 0.05$) are represented by the following symbols over the corresponding bar on the graph:
x = significantly different from 1% FBS control;
o = significantly different from pronase control;
* = significantly different from untreated P6-3 at 30 ng/ml.



0% represents 1% FBS control

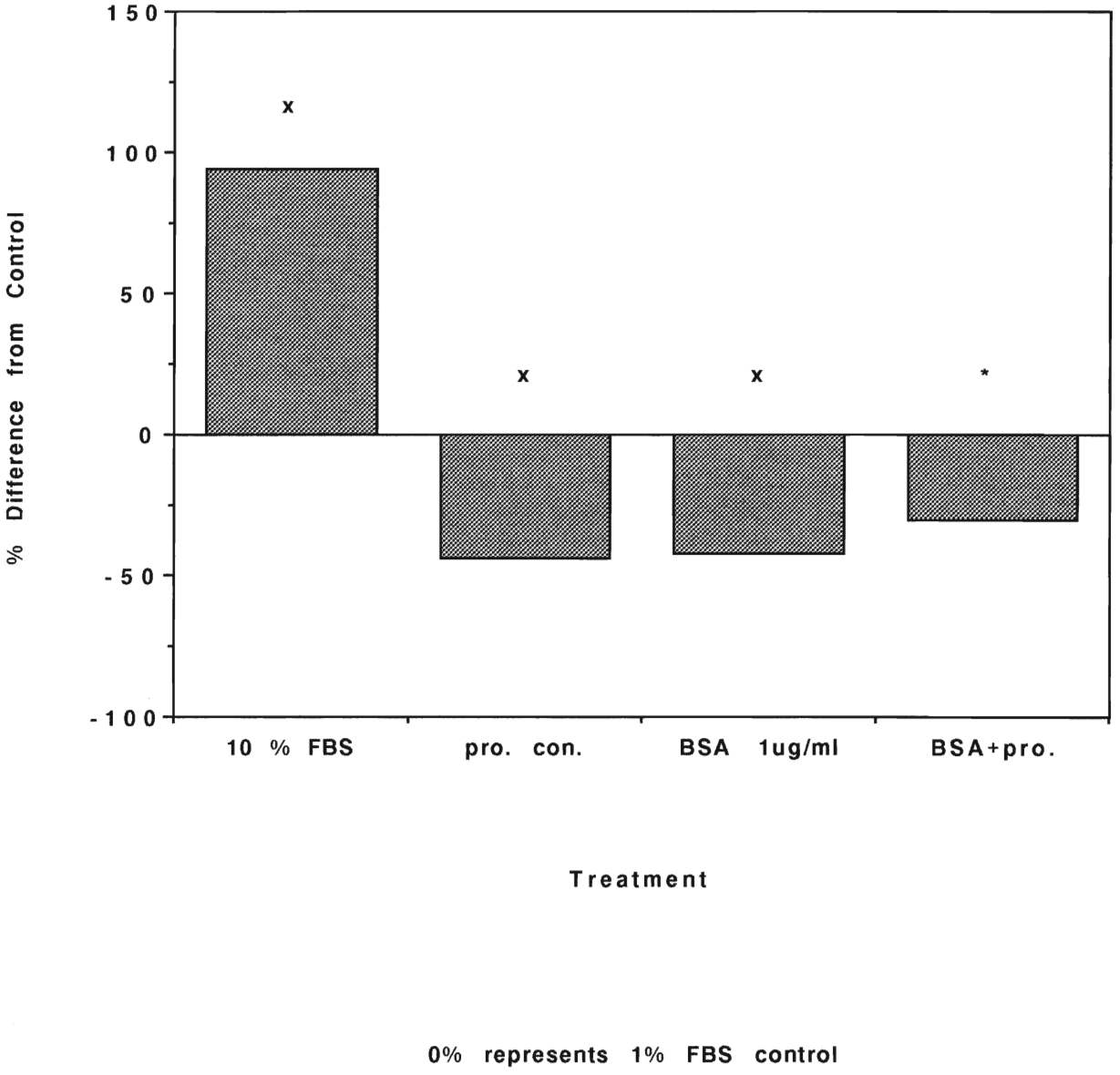
BSA at 1 µg/ml was also tested to determine the effects of this protein alone on cell proliferation. The findings of this control assay are given in Figure 28. In this assay, pronase alone caused a statistically significant 43.6% decrease in ^{3}H-thymidine uptake in the non-neuronal cells. BSA alone was also inhibitory to cell proliferation affecting a significant 42.0% decrease. Pronase digested BSA also caused a decrease in 3H-thymidine incorporation of 30.5% in the cells; this decline was to a lesser extent than caused by BSA alone. In fact, this represented a statistically significant net increase in cell growth compared to the level accomplished by BSA alone. For those cells tested with pronase treated BSA, the final concentration of BSA applied to the cells was 1 µg/ml. In contrast, 10% FBS significantly stimulated a 93.6% increase in ^{3}H-thymidine incorporation in these cells.

F. Fast atom bombardment mass spectrometry of rp-HPLC fraction #7 and various other molecules

Fast atom bombardment mass spectrometry (FAB-MS) was a technique employed to ascertain the molecular weight and other characteristics of the chemical composition of rp-HPLC fraction #7. Since previous work had indicated that the active fraction isolated from the chick embryo brains was a peptide of an estimated molecular weight of 1,500 Da. (Carlone *et al.*, 1987), the system was initially tested with a peptide of similar molecular weight. Bombesin, at 1,619.8 Da, was chosen for this purpose; its amino acid sequence has been shown to be pGlu-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2 (Woll and Rozengurt, 1989). Bombesin was dissolved in either 5% aqueous glycerol (for flow probe) or glycerol alone (for mega probe). It did not readily go into solution, but instead formed a milky white liquid. The bombesin sample was introduced into the instrument in two ways: using a flow probe and on the surface of a mega probe. Positive-ion spectra were

Figure 28. Effect of Pronase Treated BSA on ^{3}H-thymidine Incorporation in Cultured Chick Embryo Brain Non-neuronal Cells.

BSA was incubated with pronase and tested at a P6-3 concentration of 1 µg/ml (BSA + pro.); controls of distilled H_2O incubated with pronase (pro. con.) and untreated BSA at 1 µg/ml (BSA 1µg/ml) were also tested. Data are presented as percent difference from the 1% FBS control for each of the treatments tested and the 10% FBS control. n = 4 for the 1% and 10% FBS controls and the pronase control; n = 6 for the untreated BSA and the pronase treated BSA. Second subculture cells were used. (BSA = bovine serum albumin; FBS = fetal bovine serum) Statistically significant differences ($p \leq 0.05$) are represented by the following symbols over the corresponding bar on the graph:
x = significantly different from 1% FBS control;
* = significantly different from untreated BSA at 1 µg/ml.



0% represents 1% FBS control

collected. The first inlet system was successful in yielding a pseudo-molecular ion at m/z 1,621 (bombesin + H^+), but little amino acid sequence information. However, sample introduction via the mega probe not only produced the pseudo-molecular ion at m/z 1,621, but also useful sequence data (Figure 29). Amino acid sequence specific information may be obtained using FAB-MS by virtue of the fact that fragmentation generally occurs at the peptide bond of peptides and proteins. A peak at the appropriate fragment ion molecular weight thus appears in the spectrum. Beginning at the pseudo-molecular ion, it was possible to determine the first six amino acid segment of bombesin from the carboxy-terminus (pGlu-Gln-Arg-Leu-Gly-Asn-). In this manner, it was also possible to elucidate the final seven amino acid portion of the peptide from the amino-terminus (Met-Leu-His-Gly-Val-Ala-Trp-). Charting the fragmentation of these sequence specific sections of bombesin was facilitated by the prior knowledge of this peptide's amino acid sequence.

In contrast, the FABMS of rp-HPLC fraction #7 was not so straightforward. Like bombesin, this mitogen was reconstituted in 5% aqueous glycerol (for flow probe) or glycerol alone (for mega probe). However, this substance readily dissolved to yield a clear, colourless liquid. The sample rp-HPLC fraction #7 was also introduced into the mass spectrometer via both the flow probe and the mega probe. The flow probe was used in two attempts to acquire a mass spectrum. Positive ion spectra were collected. Fragmentation of the sample occurred, but no clearly identifiable molecular ion or fragmentation pattern resulted. The largest ions apparent were at m/z 1522 in the first spectrum and at m/z 1248 in the second spectrum. Work with the mega probe also did not reveal a definitive molecular ion. From Figure 30, it is evident that the ion of largest molecular weight is at m/z 1610. Fragmentation patterns are not readily distinguishable and amino acid sequence ions (should CBGF be a peptide) are not clearly visible as they were for bombesin. Three

Figure 29. Positive-ion FAB Mass Spectrum of Bombesin.

The sample was dissolved in glycerol and introduced via a mega probe. Amino acid fragmentation from both the carboxy-terminus and amino-terminus of the peptide are indicated.

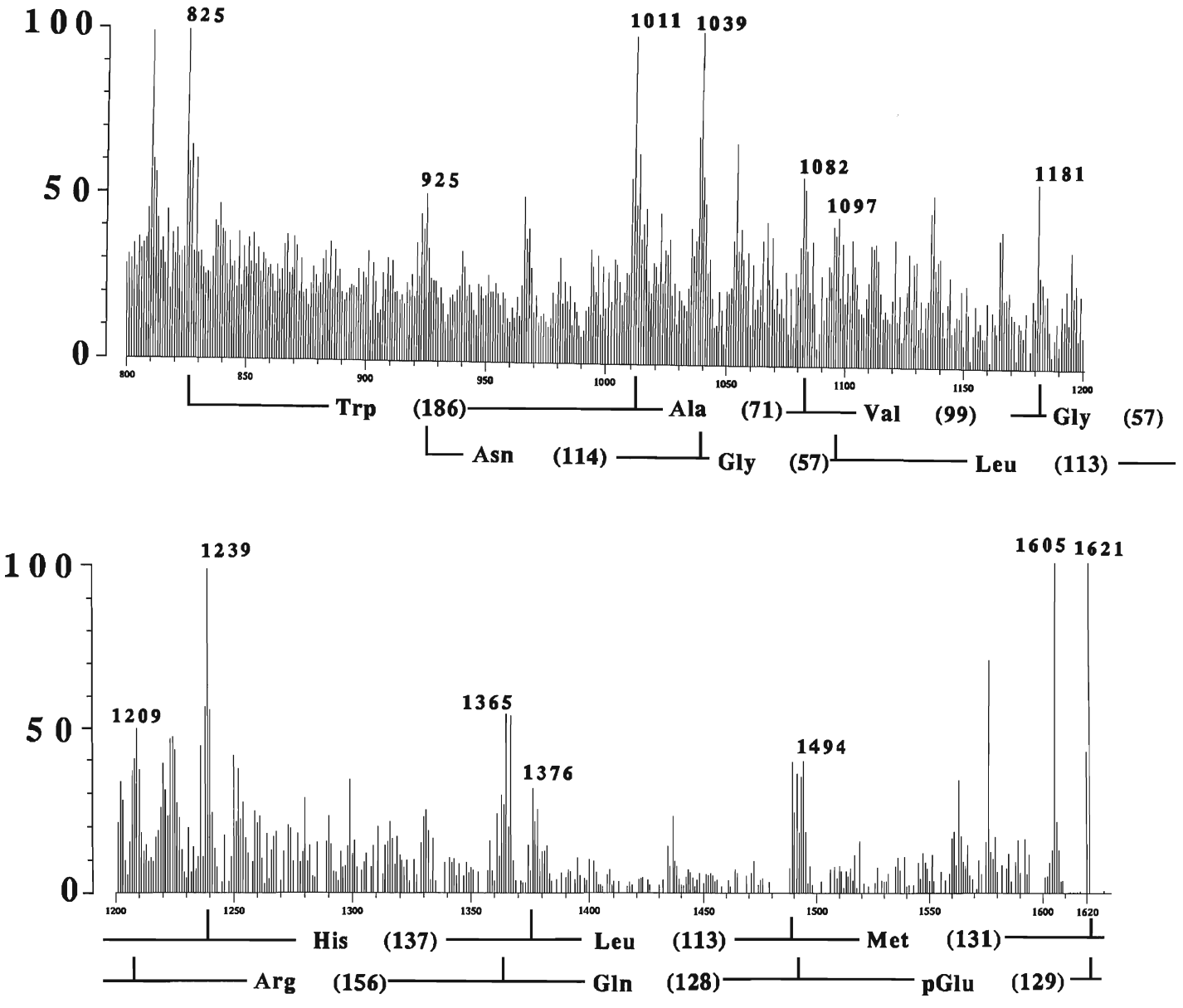
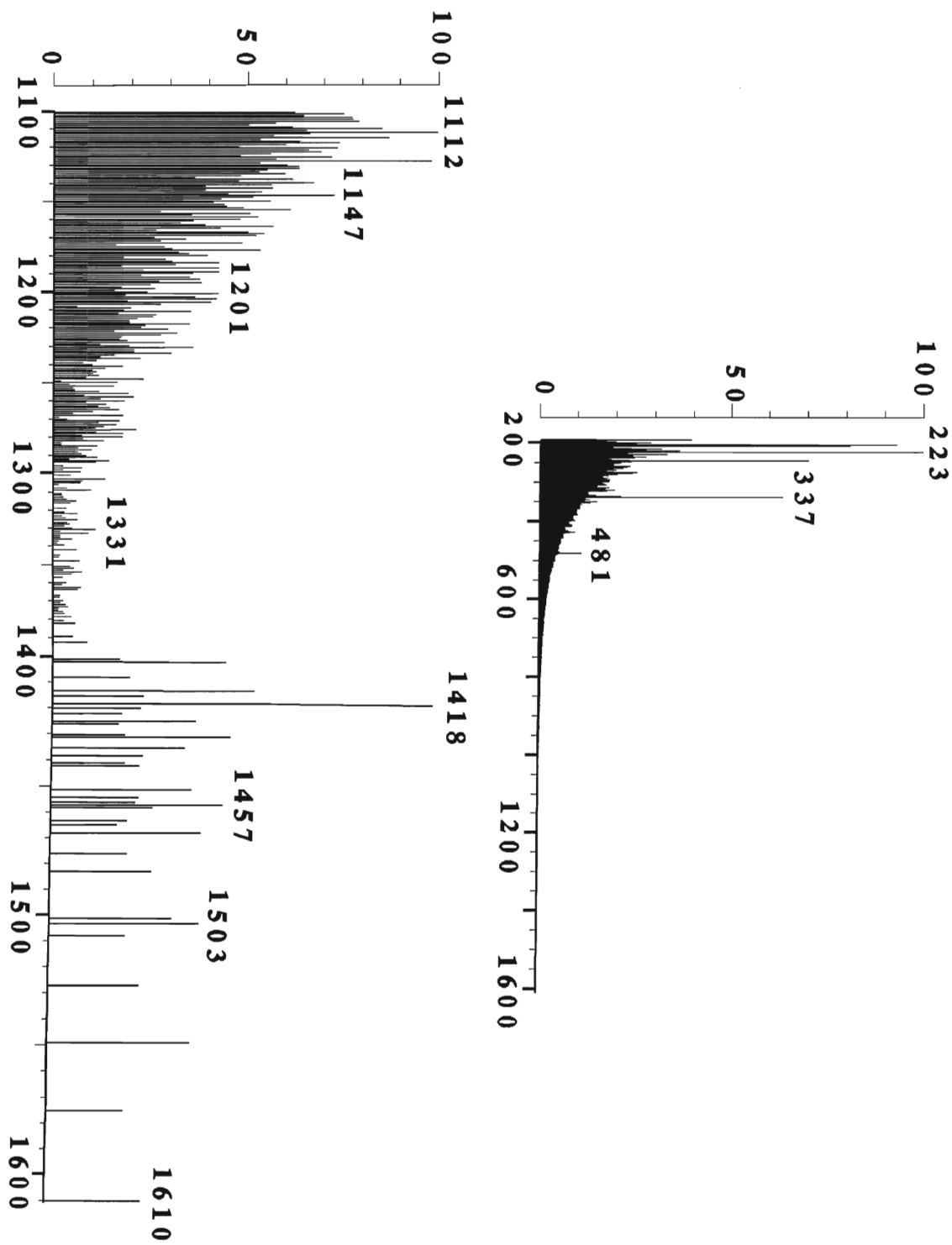

100
50
0
825
925
1011
1039
1082
1097
1181
800
850
900
950
1000
1050
1100
1150
1200
Trp
(186)
Ala
(71)
Val
(99)
Gly
(57)
Asn
(114)
Gly
(57)
Leu
(113)
1239
1209
1365
1376
1494
1605
1621
1200
1250
1300
1350
1400
1450
1500
1550
1600
1620
His
(137)
Leu
(113)
Met
(131)
Arg
(156)
Gln
(128)
pGlu
(129)

Figure 30. Positive-ion FAB Mass Spectrum of rp-HPLC Fraction #7.

The sample was dissolved in glycerol and introduced via a mega probe.


100
50
0
223
337
481
200
600
1200
1600
100
50
0
1112
1147
1201
1331
1418
1457
1503
1610
1100
1200
1300
1400
1500
1600

particularly abundant ions are present at low molecular weight. At m/z 223, an ion of two glycerol molecules with a potassium atom can be seen. The ion at m/z 337 is representative of a glycerol trimer with associated potassium and sodium atoms. The identity of the ion at m/z 481 was deduced to be three glycerol molecules with five accompanying sodium atoms and one phosphate (PO_4) group. The sample had been dissolved in a glycerol matrix. Glycerol forms aggregates and the glycerol spectrum is often visible when the spectrum of the sample of interest is weak. In the purification of rp-HPLC fraction #7 various buffers were used. Some of the metal ions of these salts have evidently associated with the sample and with its glycerol matrix. Ions with these associated metal atoms do not appear in the FAB mass spectrum of glycerol alone. The three ions at m/z 223, 337, and 481 were also present in the mass spectra of rp-HPLC fractions #10 and #11, much later eluting fractions than rp-HPLC fraction #7. This would indicate that these ions are matrix-related, supporting the aforementioned identifications.

Since rp-HPLC fraction #7 behaved differently than the bombesin sample under identical FAB-MS conditions and since other researchers had isolated both GMP and AMP from the 18-day chick embryo brain (Kim *et al.*, 1991; Rathbone *et al.*, 1992a), the positive-ion FAB mass spectra of a variety of nucleotides and other molecules were determined. The following molecules were analyzed by FAB-MS: 5'-GMP, cGMP, 5'GDP, 5'-GTP, ddGTP (2,3-deoxy GTP), 5'-AMP, cAMP, 5'-ADP, 5'-ATP, ddATP, 5'-TMP, ddTTP, ddCTP, glucose, sorbitol, and starch. None of these samples dissolved as readily in glycerol as rp-HPLC fraction #7. Moreover, the mass spectra did not share similar features with the rp-HPLC fraction #7 mass spectrum. Each nucleotide displayed a strong pseudo-molecular ion at an m/z value of the molecular weight + 1; no abundant ions at any of these m/z values were evident in the mass spectrum of rp-HPLC fraction #7.

DISCUSSION AND CONCLUSIONS

Optimization of Cell Culture and Bioassay Conditions

This research has established the basic growth requirements of cultured non-neuronal cells from the 10-day chick embryo brain. In addition, the three bioassay parameters, duration of quiescence, stimulation, and ^{3}H-thymidine labeling, were determined for these non-neuronal cells *in vitro*. A preliminary attempt to define the cell culture composition was also undertaken.

The non-neuronal cells employed in this study grew best on plastic tissue culture dishes coated with the extracellular matrix component collagen as opposed to uncoated dishes. The cells proliferated rapidly on this substrate, with primary cultures reaching confluence after approximately three days; while some other research groups do not use collagen-coated dishes when culturing astroglial cells, their cultures grow more slowly, not reaching confluence until 7 to 12 days (Courtin *et al.*, 1990; Huff and Schreier, 1990; Jensen and Chiu, 1991).

The ability of non-neuronal cells to respond to a proliferative stimulus (10% FBS) was found to be dependent on the number of subcultures the cells had undergone. Thus, second subculture cells were determined to be more responsive than first subculture cells. While no statistical difference was detected between second and third subculture cells, the older cells demonstrated a general decrease in responsiveness. Since only those assays in which a significant difference in ^{3}H-thymidine incorporation between treatment with 1% and 10% FBS were utilized, an insufficient number of fourth subculture cells remained to be compared to third subculture cells; none the less, this in itself was an indication that these older cells were less responsive to 10% FBS than their second and third subculture counterparts from the same cell preparation. Other

studies have shown that, during serial subcultivation, the percentage of cells participating in DNA synthesis decreases, the average cell cycle time increases, primarily as a result of an increase in G_1, and that, consequently, senescent cells are thought to be blocked in late G_1 or at the G_1/S border (Cristofalo *et al.*, 1989). Rathbone *et al.* (1992c) demonstrated that the rate of proliferation of cultured primary cells decreased as they aged *in vitro*, with chick brain astrocytes exhibiting this decline earlier than meningeal fibroblasts or human endothelial cells. Furthermore, as the number of serial subcultivations increases, the sensitivity of cells to growth factors diminishes; this was observed for chick brain astrocytes, meningeal fibroblasts, and human endothelial cells in response to purine nucleosides/nucleotides (Rathbone *et al.*, 1992c) and for rat Schwann cells in response to glial growth factor (Goodearl *et al.*, 1993). Late passage fibroblast cells have been thought to resist mitogenic stimulation in part because of their increased production of prostaglandin E2 to levels that inhibit growth in young cells (Cristofalo *et al.*, 1989). In addition, the number of growth factor receptors may decline for aged cells as is the case for high affinity epidermal growth factor receptors on cultured fibroblasts (Kawamoto *et al.*, 1989). The conclusions of the present study indicate that the chick non-neuronal cells are most responsive to growth factor stimulation and most effective in a bioassay when used at the second (preferably) or third subculture.

When a comparison of ^{3}H-thymidine incorporation in non-neuronal cells grown in incomplete medium containing only α-MEM, FBS, glucose and antibiotic/antimycotic and those grown in complete medium containing the aforementioned ingredients as well as the additives insulin, sodium selenate, transferrin, putrescine and progesterone was made, it was found that complete medium was more effective; thus, complete medium was employed throughout this work. The added components of the complete medium were also individually supplemented to 0.1% FBS in

incomplete medium, demonstrating that $3x10^{-8}$ M sodium selenate and 5 µg/ml insulin increased DNA synthesis, while $2x10^{-8}$ M progesterone decreased it. Since the results of the individual components were only compared to 0.1% FBS and 10% FBS in incomplete medium on the same culture plate, it would also be informative to compare these findings to 0.1% FBS in complete medium in a future study. The present results indicate that the medium supplements are more effective when used in conjunction with each other than when employed individually; the contribution to cell stimulation by each could be additive or synergistic. The use of supraphysiological levels of insulin in this study and others supports the view that insulin's effects are mediated by the insulin-like growth factor-1 (IGF-1) receptor (Baskin *et al.*, 1988; Toran-Allerand *et al.*, 1991).

Despite the fact that other studies have employed a serum-free medium with which to render their astroglial cells "quiescent" (Carlone *et al.*, 1987; Kim *et al.*, 1991), this research has ascertained that non-neuronal cells treated with either 0% or 0.1% FBS appeared less healthy and experienced increased cell death than cells in the presence of either 1% or 10% FBS. Thus, even though use of 0.1% FBS more effectively meets the goal of quiescence, that is, to maximize the difference between cell growth in the "quiescent" state and in 10% FBS, use of 1% FBS was deemed more suitable in light of its positive effect on non-neuronal cell health.

The conditions of the growth factor bioassay on the chick non-neuronal cells had not been previously optimized. The first parameter examined was the duration of "quiescence". Since the cell culture medium contained 1% FBS for this step in the bioassay, the cells were actually at a basal level of growth rather than truly quiescent and not dividing; none the less, the goal of maximizing the difference between the cells' response to 10% and 1% FBS was achieved. Incorporation of ^{3}H-thymidine decreased over the period from 3 to 14.5 hours of "quiescence". This

represented a diminishing residual response to growth in 10% FBS, likely due to an average decreasing rate in progression through the cell cycle. After 14.5 hours, a constant basal level of growth was maintained until at least 72 hours "quiescence"; during this time, the cells were no longer reacting to the residual effects of growth in 10% FBS, but were responding to growth in 1% FBS with a stabilized, constant rate of movement through the cell cycle. Thus, it was during this period that the difference between growth in 10% and 1% FBS had reached a maximum. Twenty-four hours "quiescence" was chosen as a convenient time which fell within this range, and was employed in all subsequent bioassays.

The second bioassay constraint to be examined was the length of cell growth stimulation prior to the addition of a radioactive label. In the presence of 10% FBS, the non-neuronal cells had an increasing rate of ^{3}H-thymidine incorporation which reached a maximum at 6 hours of "prestimulation"; this was followed by an initial rapid and then slow decline in label uptake. Thus, the optimum duration of "prestimulation" was 6 hours, or a total of 30 hours of growth stimulation, 24 hours of which included a radioactive label. This lag in response to a growth stimulus, such as 10% FBS, has been attributed in other studies to be a result of residual effects from the lengthy "quiescence" period which precedes the stimulation phase (Huff and Schreier, 1990). As a comparison, treatment of rat astrocytes with either bFGF or EGF caused an increase in ^{3}H-thymidine incorporation which maximized after 18 to 24 hours (Huff and Schreier, 1990).

Other researchers have reported the time required for cells in culture to <u>first</u> demonstrate a response to a growth stimulus following quiescence. Kim *et al.* (1991) determined that chick astrocytes needed less than 8 hours in the presence of GMP before an increase in ^{3}H-thymidine incorporation was registered. The human fibroblast cell line W1-38 entered DNA synthesis 12 hours after the addition of EGF, IGF-1 and

dexamethasone (Cristofalo *et al.*, 1989); DNA synthesis in Syrian hamster embryo cells was induced 10 to 20 hours following stimulation by 10% FBS (Futreal and Barrett, 1991). The onset of DNA synthesis could be determined for the chick non-neuronal cell system employed in this study by varying the 10% FBS stimulation time and using a small ^{3}H-thymidine labeling time. It is interesting to note the decline in DNA synthesis that takes place once the 6 hour maximum of "prestimulation" has been reached. This decrease could be due to the depletion of the growth stimulus in the medium by proliferating cells, the accumulation of toxic cellular metabolites, or a combination of the two. In any case, the "prestimulation" time should not exceed 6 hours; the previously used 24 hour "prestimulation" time was too long since it fell in the range of declining DNA synthesis.

The third aspect of the bioassay to be studied was the duration of ^{3}H-thymidine labeling. The amount of radioactive label uptake by the non-neuronal cells increased linearly in the presence of 10% FBS over a 3 to 48 hour labeling period. Due to this positive correlation between ^{3}H-thymidine incorporation and time, 24 hours was selected as a convenient duration for labeling and was used throughout this study. Other researchers have reported a similar linear relationship in their examination of ^{3}H-thymidine incorporation by chick embryo astrocytes in the presence of either 10% FBS, chick embryo brain extracts, guanine nucleotides, or adenosine nucleotides over an 8 to 30 hour labeling period (Kim *et al.*, 1991; Rathbone *et al.*, 1992a).

Since the optimization of the duration of pre-label stimulation and ^{3}H-thymidine labeling was accomplished using 10% FBS, the results obtained may be specific for treatment with this particular growth stimulus and may differ slightly for the brain extracts and nucleotides tested in this study. Future experiments pinpointing the precise bioassay parameters required by these non-neuronal cells when using chick brain

mitogens could alleviate this uncertainty.

A preliminary characterization of the composition of 10 day chick embryo non-neuronal brain cells indicated that primary 4 day subconfluent cells consisted of 6.63% GFAP positive cells. Despite the fact that this finding suggests that these cultures contain few astrocytes, the cells displayed the distinctive cobblestone morphology attributed to confluent glial precursors, particularly astroblasts (Sensenbrenner, 1977; Carlone *et al.*, 1988; Andersson *et al.*, 1994). Previously, this laboratory had observed that cultures of 10 day chick embryo brain cells with this cobblestone morphology were >90% GFAP positive (Carlone *et al.*, 1988); however, these cultures, unlike the ones employed in this study, had been passaged an unreported number of times and were confluent when they were tested one week after plating. It is therefore possible that these two situations characterize not distinct cell types, but rather different subpopulations of one cell type, tested under unique conditions. In fact, astrocyte heterogeneity has been shown in type I astrocytes isolated from anatomically distinct regions of the CNS (Wilkin *et al.*, 1990; Landis, 1994). Furthermore, marked differences in GFAP levels have been observed in astrocytes cultured from different regions of the brain (Wilkin *et al.*, 1990).

It is the differing conditions under which these cells were tested which may have caused different subpopulations of the same non-neuronal cell type to arise. One of the factors to be considered is the age of the cell culture. Similar to the research on chick embryo astrocytes (Carlone *et al.*, 1988), neo- and post-natal rat astrocytes have been observed to be 95% GFAP positive (Huff and Schreier, 1990; Jensen and Chiu, 1991); however, both of these instances represent much older cultures which remained as primary cultures for up to 10 days and were subsequently subcultured prior to GFAP testing. Kim *et al.* (1991) also reported that their older chick astrocyte cultures were over 90% GFAP

positive, but that early astroblast cultures contained a majority of cells that had not yet become GFAP positive. This result agrees with other investigations showing that astroblasts (astrocyte precursors) do not stain for GFAP (Pixley *et al.*, 1984). This may suggest that the cultures of this study were young, consisting primarily of astroblasts. Andersson *et al.* (1994) observed that the density of glial filaments in type 1 rat astrocytes dramatically increased with increasing days *in vitro*. While the morphology of the astrocytes in the present study appeared to be that of type 1 astrocytes, this trend has also been observed in type 2 astrocytes: immature type 2 astrocytes *in vitro* express low levels of GFAP, however once they mature GFAP expression increases (Lillien and Raff, 1990).

Another aspect of cell culture conditions that has been shown to play a role in GFAP expression is the cell density (Toran-Allerand *et al.*, 1991). Unlike the aforementioned 90% GFAP positive confluent astrocyte cultures of Carlone *et al.* (1988), those employed in the present work were subconfluent. Goldman and Chiu (1984) have shown that while the majority of neonatal rat astrocytes in their studies were GFAP positive regardless of the cell density, low density cells possessed a dimmer fluorescence intensity than high density cells. A general faint level of fluorescence, above those of control assays, was observed in non-neuronal cells tested in the present study; however only those cells displaying an obviously high degree of fluorescence were counted as GFAP positive cells. This possibly erroneous result could be clarified either by allowing the cells to reach confluence or by treating low density cells with colchicine to induce coiling of intermediate filament bundles, thereby making immunofluorescent staining easily visible (Goldman and Chiu, 1984). It has also been shown that low density astrocytes actually contain and synthesize less GFAP than high density astrocytes (Goldman and Chiu, 1984).

The biochemical environment of astroglial cells also influences the

level of GFAP expression. For example, acidic and basic FGF have been demonstrated to change the distribution of GFAP when supplied to astoglial cells *in vitro* (Weibel *et al.*, 1985). The non-neuronal cells of this study were cultured in the presence of a variety of growth promoting substances including 5 μg/ml insulin. Other researchers have found that few polygonal nonprocess-bearing glial cells were stained by GFAP in organotypic cultures of embryonic mouse cerebellar astroglia exposed to similarly high insulin levels (10 μg/ml) (Toran-Allerand *et al.*, 1991). Moreover, the concentration of progesterone used in the culture medium of the current study was 100-fold lower than that employed by Carlone *et al.* (1988); although the published concentration of progesterone utilized by Carlone *et al.* (1988) was the same used in this study ($2x10^{-8}$ M), the actual concentration used by these researchers was $2x10^{-6}$ M. This difference in the culture media of these two studies may be sufficiently great to alter the degree of GFAP expression.

Isolation and Purification of Chick Brain Growth Factor

This research has expanded and improved the isolation and purification scheme for chick brain growth factor first outlined by Carlone and his colleagues (Carlone and Rathbone, 1985; Carlone *et al.*, 1987). In the current study, CBGF was partially purified by ultracentrifugation, ultrafiltration through Amicon PM-30 and YM-2 membranes (nominal molecular weight cut-offs of 30,000 Da. and 2,000 Da., respectively), size exclusion chromatography through a Biogel P6 column, and C_{18} rp-HPLC. While a number of biological activities were present in the rp-HPLC fractions, the most potent mitogen for cultured non-neuronal cells from the 10 day chick embryo brain resided in a fraction (rp-HPLC fraction #7) found to be as effective as 10% FBS at stimulating ^{3}H-thymidine incorporation into these cells when tested at 10 ng/ml. This fraction

represented a 943 fold purification of the active material over that of the crudely purified PM-30 filtrate. The CBGF isolated in this work had a high specific mitogenic activity in the non-neuronal cell bioassay, causing a half-maximal stimulation of ^{3}H-thymidine (ED_{50}) into these cells at a concentration of 5.3 ng/ml. This value is comparable to the one previously reported for CBGF (ED_{50} = 5 ng/ml; 3 nM), purified to apparent homogeneity using a slightly different protocol, but also to the level of C_{18} rp-HPLC (Carlone *et al.*, 1987). The fact that CBGF is active at a physiologically relevant concentration is evidenced by the comparable ED_{50} values exhibited by other growth factors and neuropeptides such as PDGF (1 ng/ml; Raines and Ross, 1985), aFGF (~40 pg/ml; Thomas *et al.*, 1984), vasopressin (1 ng/ml; Rozengurt *et al.*, 1979), and bombesin (1 nM; Rozengurt and Sinnet-Smith, 1983).

The CBGF partially purified in this research was observed to be as biologically active as 10% FBS when tested on chick embryo non-neuronal cells *in vitro* ; this was also the finding reported by Carlone *et al.* (1987) for their homogeneous preparation. CBGF appears to be a particularly potent mitogen. By comparison, EGF stimulates only one half and bFGF merely one sixth as much ^{3}H-thymidine incorporation in cultured rat astrocytes as 10% FBS (Huff and Schreier, 1990).

The chick embryo brain non-neuronal cells exhibited a bell-shaped concentration dependence for the CBGF isolated in the current study, much like that observed previously by Carlone and Rathbone (1985). Such a bell-shaped concentration dependence was also reported by Choo *et al.* (1978; 1981) when these researchers examined the stimulation of protein synthesis in cultured blastema explants from the regenerating amphibian limb treated with a factor isolated from chick brains. Furthermore, both the CBGF isolated in this research and the factor isolated by Choo *et al.* (1978) are biologically active over a relatively narrow concentration range: CBGF, between 1 ng/ml and 100 ng/ml, and Choo's factor, between

0.5 mg/ml and 1.5 mg/ml. The findings of Choo and his colleagues suggest that their factor was a potential candidate for the trophic factor(s) involved in blastema cell proliferation during the nerve-dependent limb regeneration of Urodele amphibians. CBGF (10 ng/ml) has also been found to be mitogenic for cultured forelimb blastemas from the adult newt, *Notophthalmus viridescens*, causing an average 3.6-fold increase in the percentage of ^{3}H-thymidine-labelled blastema nuclei when compared to control values (Carlone *et al.*, 1987). Moreover, like the factors isolated from nerve tissue in previous attempts to purify the trophic factor involved in limb regeneration (Singer *et al.*, 1976; Choo *et al.*, 1981), CBGF activity lacks species specificity since a similar preparation from adult newt brain is also mitogenic for cultured blastemas (Carlone and Rathbone, 1985). Therefore, these findings point to a possible role for newt CBGF in the nerve-dependent stimulation of blastema cell proliferation during amphibian limb regeneration.

The CBGF isolation procedure, as previously described (Carlone and Rathbone, 1985; Carlone *et al.*, 1987), involved the ultrafiltration of 18-day chick embryo brain homogenates through a series of Amicon filters of decreasing porosity. The retentate of the final filter in this sequence, the UM-05 membrane (nominal molecular weight cut-off of 500 Da.), contained the biologically active fraction. However, this membrane, made of a blend of polyelectrolytes, is no longer manufactured; the replacement membrane, YC-05, is made of cellulose and has a nominal molecular weight cut-off of 300-400 Da. Prior to this study, it was found that the YC-05 membrane did not retain the active fraction. Since the estimated molecular weight of CBGF (1,500-2,000 Da.) was greater than the molecular weight cut-off of the YC-05 filter, making it unlikely that CBGF would pass through the membrane, it was speculated that the active fraction was irreversibly bound to the new membrane. As a result, the filtrate collected in the purification step preceding the use of the UM-05 membrane was

applied to a Biogel P6 size exclusion column (with a molecular weight cut-off of 6,000 Da.) from which a biologically active fraction was collected. Use of a DEAE anion exchange column, as described by Carlone *et al.* (1987), had been discontinued in a previous study (Seifried, honours thesis, 1988). Purification of the active material proceeded with rp-HPLC (Carlone *et al.*, 1987). Since, several biological activities have been identified in this system (Carlone *et al.*, 1987), the changes to the isolation and purification protocol described in this study may have resulted in the isolation of a mitogen different from CBGF.

Variations in the non-neuronal cells' response to 10% FBS were seen throughout the course of this study. A factor in these variations, namely the number of subcultures which the cells have undergone, has already been discussed. However, differences in cell responsiveness were observed even in cells from the same subculture; this observation has also been noted by other researchers working with cultured chick embryo astrocytes (Rathbone *et al.*, 1992b). Throughout the course of this research, all test wells were seeded at the same density. However, the length of time from the seeding of a plate to the onset of a bioassay varied slightly from plate to plate. Thus, although the individual wells of any given culture plate all had equivalent numbers of cells at the start of each bioassay, the density of cells from one plate to another was different. For this reason, it was necessary to include control wells containing 1% FBS and 10% FBS in each bioassay plate and to express all data as the percent difference from 1% FBS.

The difference in cell number in each bioassay plate is one factor which can account for the apparent variation in cell responsiveness. Less EGF stimulation has been found to occur in high versus low density cultures of the human glial cell line (Westermark, 1976); furthermore, bFGF potency has also been observed to be negatively correlated with plating density when tested on cultures of CNS neurons (Knusel *et al.*,

1989). Since the non-neuronal cells of the current study grow in a monolayer, one might argue that growth stimulated high density cultures would be prevented from further growth once the cells are confluent, thereby explaining the results reported by Westermark (1976) and Knusel *et al.* (1989). However, this supposition does not affect the validity of the results obtained for cells tested with a test fraction, since 10% FBS stimulated ^{3}H-thymidine incorporation into the cells to a greater degree. Moreover, the situation is more complex than a simple contact inhibition effect, as evidenced by the fact that researchers have found the number of surface bFGF receptors to decrease with increasing cell density in a nontransformed cell line (Veomett *et al.*, 1989). It has also been suggested that in high density cultures added factors, like bFGF, are more rapidly removed from the medium by a variety of mechanisms, such as specific and nonspecific binding, causing the effective factor concentration in the medium to quickly decline (Knusel *et al.*, 1990). Other studies contrast with these, finding greater EGF stimulation in high density neonatal rat astrocyte cultures; the authors attributed these results to a possible starvation effect for EGF in more confluent cultures or a synergistic effect from a different unknown factor produced in greater quantities at higher cell densities (Huff and Schreier, 1990). Each of these examples points to a variable response to the same factor by lower and higher density cultures.

Characterization of CBGF's Biochemical Characteristics

UV absorbance spectra, rp-HPLC profiles, and mass spectra comparing rp-HPLC fraction #7 and commercially available 5'-GMP have all clearly shown that the partially purified CBGF found in rp-HPLC fraction #7 is not 5'-GMP. Moreover, 5'-GMP was tested in the non-neuronal cell bioassay over the concentration range 0.005 - 500 μM and, unlike rp-HPLC fraction #7, was not found to be biologically active.

The findings of the present study contrast with those reported by Kim *et al.* (1991). These researchers have isolated the most active of several fractions from the 18-day chick embryo brain and determined it to be 5'-GMP. They found that this fraction, as well as commercially available 5'-GMP, stimulated ^{3}H-thymidine incorporation into cultured 10-day chick embryo brain astrocytes in a dose-dependent fashion with a maximal activity at 50 μM (Kim *et al.*, 1991). In contrast, the research presented in this thesis has indicated that 50 μM 5'-GMP is inhibitory to chick embryo brain non-neuronal cell division.

The findings of Kim *et al.* (1991) do not preclude the fact that other activities reside in the chick embryo brain; indeed, these researchers noted the presence of 5'-AMP in addition to other mitogenic fractions (Rathbone *et al.*, 1992a,b), but found the fraction identified as 5'-GMP to be the most stimulatory to ^{3}H-thymidine incorporation under their bioassay conditions. Others have reported the existence of peptide growth factors in the embryonic chick brain (Choo *et al.*, 1978; Carlone *et al.*, 1987). While the results of the current study do not support the identification of rp-HPLC fraction #7 as 5'-GMP, this nucleotide may be a component of the crude chick embryo brain homogenate, albeit a biologically inactive one for the chick non-neuronal cells. Previous NMR studies have suggested that two CBGF rp-HPLC fractions, one inactive and the other inhibitory, contained nucleosides/nucleotides (M. Seifried, honours thesis, 1988).

While the factor isolation method and bioassay conditions employed in this research and by Kim *et al.* (1991) are similar, a number of differences exist which may explain the contradictory results obtained. The first of these differences is the use of the UM-O5 replacement membrane, the YC-05, by Kim *et al.* (1991); using this membrane, these researchers were successful in obtaining an active fraction in the retentate. Since no biologically active material could be isolated from the retentate in studies preceding the current thesis research, a variation in the isolation

procedure was adopted in which the YM-2 filtrate was directly applied to a Biogel P6 size exclusion column. In the study by Kim *et al.* (1991), the YC-05 retentate was applied to a DE-52 anion exchange column for further purification; a similar step, previously employed by Carlone *et al.* (1987), was eliminated in a prior study. This may suggest that these alternate protocols result in the purification of different active fractions.

Moreover, in the bioassay conditions employed in this thesis study, non-neuronal cells were rendered quiescent in medium supplemented with 1% FBS for 24 hours prior to the addition of various test fractions. However, Kim *et al.* (1991) rendered their ten-day chick embryo brain astroblasts quiescent in medium containing 0% FBS for 72 hours. When the ten-day chick embryo brain non-neuronal cells of the present study were incubated with medium containing 0% FBS, many died while the remaining cells appeared unhealthy; for this reason, medium supplemented with 0% FBS was deemed unsuitable for non-neuronal cell maintenance and 1% FBS was used. Furthermore, in the current research it was found that the optimal length of time for incubating the cells with the test factor prior to the addition of ^{3}H-thymidine was 6 hours. In contrast, Kim *et al.* (1991) utilized a preincubation time of 24 hours; it is unknown whether this time was optimized. These differences in the bioassay conditions may indicate that either different cell types or subpopulations of the same cell type are being selected for in these two studies.

Kim *et al.* (1991) reported that the cells which they used were GFAP positive, and so identified them as astroblasts. On the surface, this fact would seem to support the notion that the current research project and that of Kim *et al.* (1991) dealt with different cell types, since young primary (4 day) non-neuronal cells of the present study were determined to be largely GFAP negative, and therefore could not be identified as astroblasts/astrocytes. However, as previously mentioned, Kim *et al.* (1991) conducted GFAP testing on older cells (7 days after subculturing),

while cultures used for bioassay contained a majority of cells which had not yet become GFAP positive. As discussed in a previous section, the non-neuronal cells of this thesis work could simply be astroblasts which, like the bioassay cells employed by Kim *et al.* (1991) have not yet become GFAP positive. The differing results observed may then be attributable to the presence of various subpopulations of astroblasts which arose from the alternative bioassay conditions used.

While the data obtained in this thesis research indicates that the partially purified CBGF found in rp-HPLC fraction #7 is not 5'-GMP, the identity of the active material is still to be determined. The UV absorbance spectrum of rp-HPLC fraction #7 revealed a peak at 257.5 nm, but none at 280 nm suggesting the possibility of the presence of a nucleoside/nucleotide in the sample (Scopes, 1982). This fact, in addition to findings by other researchers showing that 5'-AMP can be isolated from the 18 day chick embryo brain, albeit as the least biologically active fraction, and that it is mitogenic for chick embryo brain astrocytes (Rathbone *et al.*, 1992a), prompted an investigation of the characteristics of commercially available 5'-AMP under the conditions employed throughout this study.

While 5'-AMP and rp-HPLC fraction #7 share a UV absorbance maximum at 257.7 nm, under identical rp-HPLC elution conditions 5'-AMP elutes much later than rp-HPLC fraction #7; furthermore, their FAB mass spectra are dissimilar. Moreover, when assessed in the chick embryo brain non-neuronal cell bioassay, 5'-AMP had no statistically significant effect on ^{3}H-thymidine incorporation in these cells at any of the concentrations tested (50 μM, 5 μM, 0.5 μM, and 0.005 μM).

The fact that 5'-GMP and 5'-AMP were found to elute later than rp-HPLC fraction #7 under identical rp-HPLC elution conditions has indicated that rp-HPLC fraction #7 does not contain these nucleotides. It is unlikely that the retention time of these nucleotides would be altered if

they were part of a mixture of molecules, such as the one represented by rp-HPLC fraction #7 (J. Miller, personal communication). Other researchers have isolated 5'-GMP from the 18 day chick embryo brain (Kim *et al.*, 1991); however, the elution time of this nucleotide (both purified from the chick embryo brain and commercially obtained) was unchanged from its elution time when a component of the partially purified mixture.

In addition to 5'-GMP and 5'-AMP, cAMP and cGMP were also tested in the non-neuronal cell bioassay and were examined by positive-ion FAB mass spectrometry. While cGMP caused a statistically significant increase in ^{3}H-thymidine incorporation over control at 5.45 nM and 0.545 nM in these cells, its pseudo-molecular ion (at an m/z value of the molecular weight + 1) was absent from the mass spectrum of rp-HPLC fraction #7. Therefore, the mitogenic activity of cGMP on the chick non-neuronal cells is unrelated to that of rp-HPLC fraction #7. Indeed, cGMP has been implicated as having a role in cell proliferation (Globus *et al.*, 1987). cAMP had no effect on the non-neuronal cells; its mass spectrometry data is inconsistent with its identification as a component of rp-HPLC fraction #7.

The lack of a UV absorbance peak at 280 nm, a region typically used to monitor the presence of peptides and proteins in a sample, does not necessarily preclude identification of the active component of rp-HPLC fraction #7 as a peptide/protein. The amino acids absorbing at this wavelength are tyrosine and tryptophan; a small peptide, as CBGF (approximately 1,500-2,000 Da.) appears to be as indicated by this study and others (Carlone *et al.*, 1987), might entirely lack these residues, thereby in turn lacking the appropriate peak in its UV spectrum. Indeed, preliminary amino acid analysis confirms that CBGF does not contain tyrosine or tryptophan (Carlone *et al.*, 1987). The existence of a UV absorbance maximum in a region generally associated with absorption by nucleic acids may indicate that rp-HPLC fraction #7 contains nucleosides/nucleotides, albeit not 5'-GMP or 5'-AMP. Although the

biologically active material may be wholly or partially comprised of nucleic acids, these molecules may merely represent a contaminant in the partially purified rp-HPLC fraction #7 where the active component is a peptide. Previous homogeneous preparations of CBGF also displayed UV absorbance maxima at 260 nm, but not at 280 nm (Carlone, personal correspondence). Alternatively, the CBGF peptide may contain the amino acid phenylalanine which absorbs UV light at 257.5 nm (Greenstein and Winitz, 1961); this possibility is, however, not supported by preliminary amino acid analysis which shows that CBGF has no phenylalanine residues (0.3 residues/mole observed), based on an estimated molecular weight of 1,500 Da. (Carlone *et al.*, 1987).

Interestingly, when P6-3 was subjected to non-specific proteolytic digestion using pronase, its activity in the chick embryo brain non-neuronal cell bioassay was greater than that of untreated P6-3. Pronase alone caused a small increase in ^{3}H-thymidine incorporation in the cells, and while this was not a statistically significant difference from the 1% FBS control, it presents the possibility that the increase in DNA synthesis produced by the pronase treated P6-3 merely represents the additive effects of pronase alone together with P6-3 alone. However, pronase treated P6-3 elicited an additional 27.7% ^{3}H-thymidine incorporation over what would be expected from a simple additive effect. Moreover, in other bioassays, the pronase control was seen to cause a slight decrease in radiolabel uptake.

If pronase treatment indeed increases the activity of P6-3, the question as to the identity of CBGF is further complicated. Previous studies showed that trypsin affected a decrease in CBGF (UM-05 retentate fraction) activity, indicating that this factor is a peptide (Carlone and Rathbone, 1985). The current pronase data raises a number of alternative possibilities for the composition of CBGF. One of these possibilities is the presence of a protein having an inhibitory effect on ^{3}H-thymidine

incorporation. This protein may be a component of the mixture of molecules making up the P6-3 fraction or may be bound to the active molecule. In light of the fact that higher concentrations of rp-HPLC fraction #7, in addition to other rp-HPLC fractions, cause a decrease in ^{3}H-thymidine uptake, the presence of an inhibitor is further supported. Pronase digestion of this inhibitor would permit the full biological activity to be realized. In order to reconcile this scenario with previous CBGF data stating that the active component is a protein (Carlone and Rathbone, 1985; Carlone *et al.*, 1987) and with UV absorbance data, one might envision a molecule consisting of a protein protected by a nucleoside/nucleotide moiety. Either both components or only the protein constituent may be required for biological activity. Alternatively, the activity isolated in this thesis study may simply be chemically different from the CBGF purified in the past (Carlone *et al.*, 1987), and may solely consist of nucleosides/nucleotides; however, it is clear that the mitogenic activity is not caused by GMP or AMP.

The existence of biologically active molecules composed of polypeptides complexed with polynucleotides has been previously documented (Folkman *et al.*, 1971; Wissler *et al.*, 1988). Folkman *et al* (1971) determined that a tumour cell-derived angiogenesis factor (TAF) was composed of 25% RNA, 10% protein, and 50% carbohydrate, with the remainder presumed to be lipoidal material. TAF activity could be destroyed by digestion with ribonuclease, but remained unchanged following exposure to trypsin; nonetheless, further studies implied that the protein moiety associated with RNA in TAF was essential for the factor's mitogenic activity (Folkman *et al.*, 1971). Other researchers have isolated a monocytic blood vessel morphogen for angiogenesis termed monocyto-angiotropin (MAT) which was found to be comprised of polypeptide, polynucleotide, and copper, calcium, sodium, and potassium ion portions; the polynucleotide component contained two unidentified

bases (Wissler *et al.*, 1986; 1988). The polypeptide, polynucleotide, and copper ion portions were all determined to be necessary for the expression of full bioactivity. It is interesting to note that the UV absorbance spectrum of MAT consists of a peak at approximately 260 nm. Moreover, the polypeptide component of MAT has proven to be inaccessible to amino acid sequencing by Edman degradation (Wissler *et al.*, 1986); this has also been found to be the case for CBGF (R. Carlone, personal communication).

Moreover, the existence of biologically active RNA molecules has also been proposed. Barakat *et al* (1984) isolated RNA as the active factor in bovine brain extracts stimulating chick neuroblast proliferation *in vitro* ; they, however, suggest that the activity may actually be mediated by nucleotides following RNA hydrolysis in the culture medium. On the other hand, due to the fact that extracellular fluid contains numerous proteins acting as either RNases or RNase inhibitors, researchers have hypothesized that their substrate, intact extracellular RNA, likely has a role as a short distance-short time intercellular messenger during the development of higher organisms (Benner, 1988).

Not all bioactive molecules are represented by the usual spectrum of biological compounds such as proteins and nucleotides; differentiation inducing factor (DIF-1) from the slime mold *Dictostelium discoideum* is a novel type of effector compound having the structure 1-(3,5-dichloro-2,6-dihydroxy-4-methoxy-phenyl)-1-hexanone (Morris *et al.*, 1987). Perhaps CBGF is a similar non-traditional bio-effector.

FAB-MS was undertaken to gain size and structural information about CBGF. While the FAB mass spectrum of rp-HPLC fraction #7 did not display a definitive molecular ion, and thereby reveal the molecular weight of CBGF, the ion of largest molecular weight was at m/z 1610. This is consistent with previous FAB-MS data which suggested that the molecular weight of CBGF is approximately 1600 Da. (R. Carlone, personal communication). Although commercially available bombesin yielded a

mass spectrum with a clear molecular ion and amino acid sequence fragmentation, similar sequence information, be it caused by amino acids or nucleotides, could not be gleaned from the rp-HPLC fraction #7 mass spectrum. This may be due, in part, to the fact that rp-HPLC fraction #7 had not been purified to homogeneity due to the lack of an adequate amount of this fraction. It is also possible that CBGF is a particularly difficult molecule from which to obtain a mass spectrum.

Much of the difficulty in obtaining conclusive answers regarding the structure of CBGF lies in the fact that even relatively large numbers of chick embryo brains (240) yield only small quantities of the active fraction (0.02 mg of rp-HPLC fraction #7). Proton NMR studies, while non-destructive, require a minimum concentration of 0.2 mM protein (Gadian, 1982); with an estimated molecular weight of 1600 Da., 0.16 mg of mitogen is needed. Larger isolations should permit purification to homogeneity. Exhaustive enzymatic digestion assays employing proteases, RNases, and DNases would then shed light on the biochemical nature of CBGF and the existence of a potential inhibitor. FAB-MS and NMR would yield molecular weight, sequence, and other structural information. Amino acid analysis and Edman degradation would also be performed.

In addition, the potential role of CBGF as the trophic factor involved in the nerve-dependent blastema cell proliferation phase of newt limb regeneration requires further investigation. In the past, CBGF has been shown to be a blastema cell mitogen *in vitro* (Carlone *et al.*, 1987). Moreover, a fraction partially purified from adult newt brain has biochemical characteristics identical to those of CBGF derived from chick embryo brains and is mitogenic for cultured blastema cells (Carlone and Rathbone, 1985; Carlone *et al.*, 1987). On the other hand, Rathbone and his colleagues have not reported the status of GMP and AMP as mitogens in nerve dependent blastema cell proliferation. In any case, CBGF, like all candidates for the trophic factor, must meet the four criteria outlined by

Brockes (1984): 1) it must be present in the blastema, 2) its level must decrease following denervation of the blastema, 3) it must stimulate cell division of 22/18 + cells, the blastema cell population which is dependent on the nerve for proliferation, and 4) an antibody against the factor should block its biological activity and prevent the mitogenic effects of the nerve, thereby mimicking denervation. Only following such experimentation could the role of CBGF in Urodele nerve dependent limb regeneration be assessed and determined.

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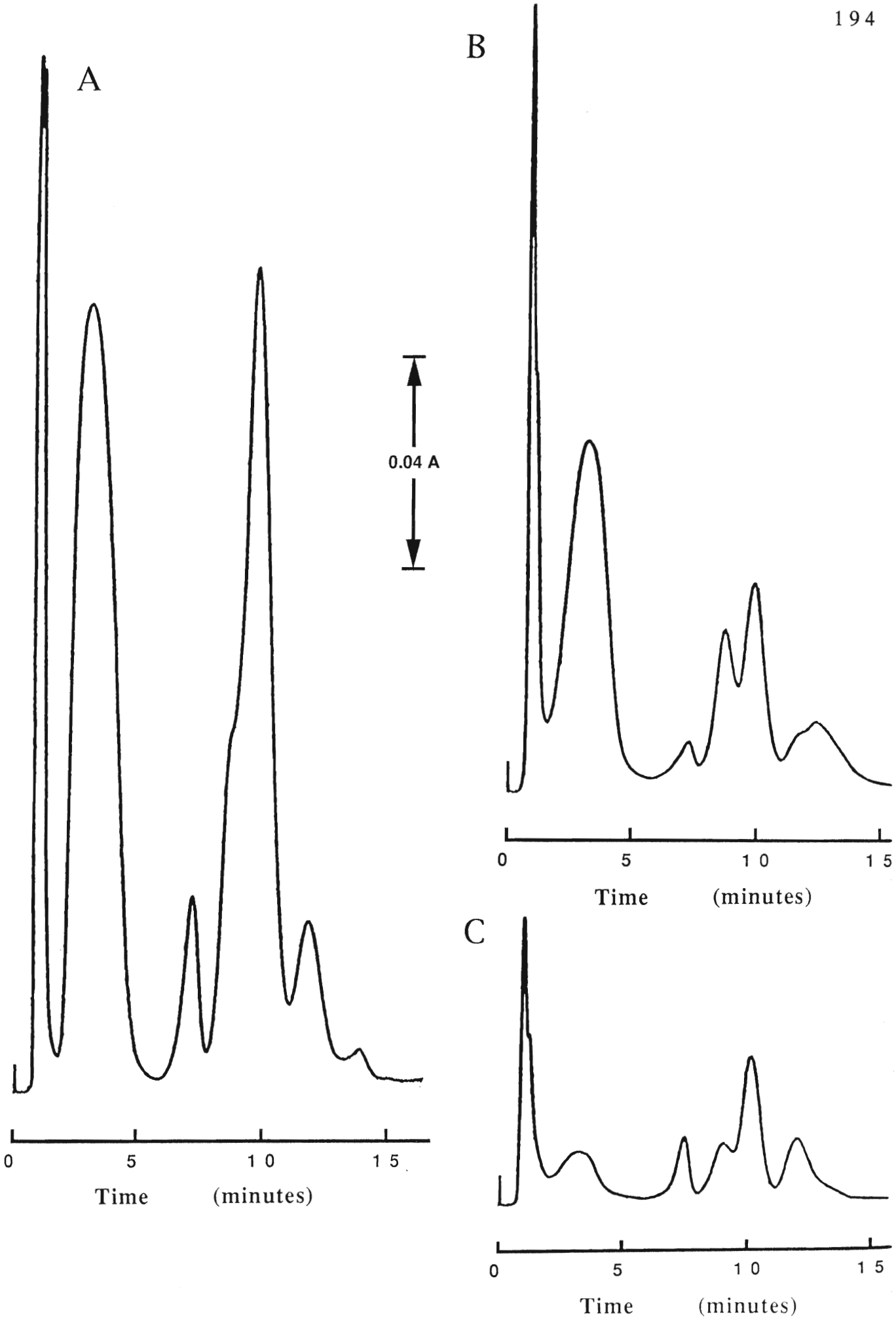
APPENDIX A

Figure A1. Anion Exchange HPLC Elution Profile of P6-3 Fractionation.

An anion exchange column was used to separate the concentrated Biogel P6-3 fraction into seven peaks, which were individually collected. The fractions were eluted with a gradient of 0.1% 0.007 M KH_2PO_4 and 0.007 M KCl (pH 4.0) to 99.9% 0.25 M KH_2PO_4 and 0.50 M KCl (pH 5.0) in 20 minutes. (HPLC = high-performance liquid chromatography)

A: The elution profile monitored at 256 nm.
B: The elution profile monitored at 229 nm.
C: The elution profile monitored at 280 nm.


A
B
C
0.04 A
0
5
10
15
Time (minutes)
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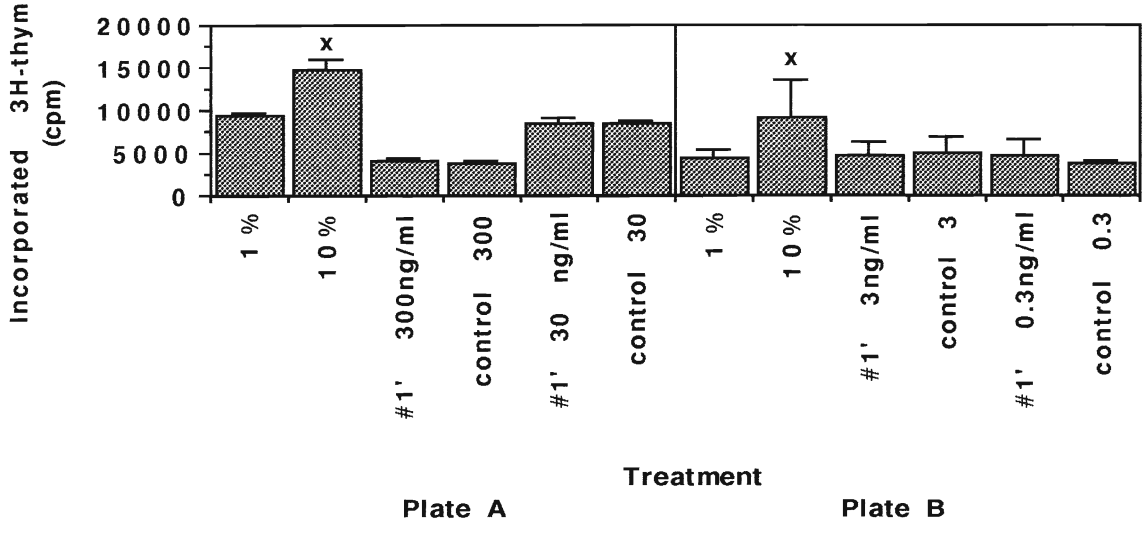
Time (minutes)

Figure A2. Effect of Anion Exchange HPLC Fractions #1, 2, and 3 on ^{3}H-thymidine Incorporation in Cultured Chick Embryo Brain Non-neuronal Cells.

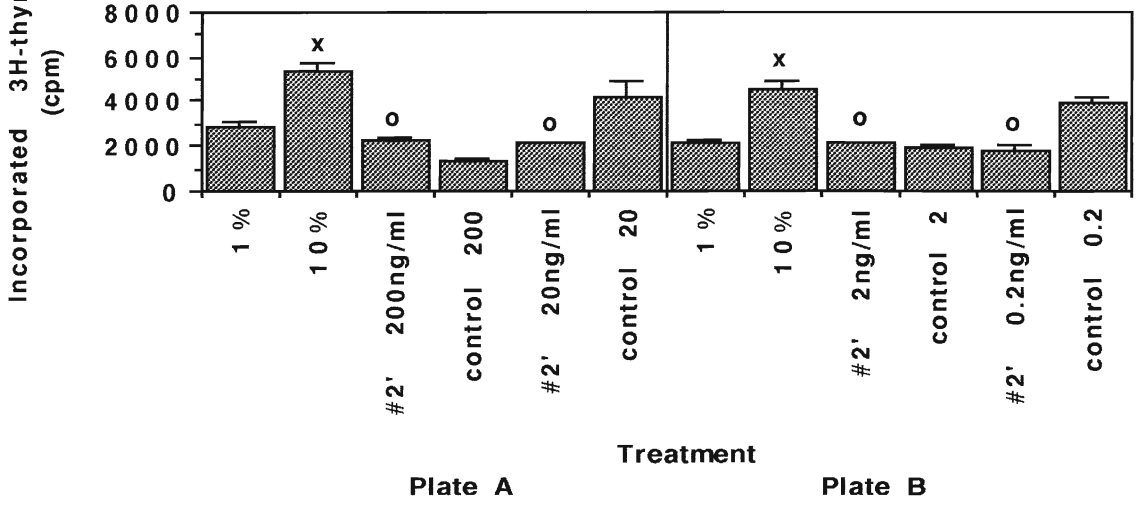
Two twenty-four well plates were assayed per fraction, represented by Plate A and Plate B. Due to the high salt concentration of the anion exchange solutions, a salt control corresponding to the KH_2PO_4 and KCl content of each fraction at every concentration assayed was also tested. Data are presented as mean incorporation of ^{3}H-thymidine in cpm. n = 4 for each treatment. [HPLC = high-performance liquid chromatography; cpm = counts per minute; 1% = 1% fetal bovine serum (FBS); 10% = 10% FBS]
Statistically significant differences ($p \leq 0.05$) are represented by the following symbols over the corresponding bar on the graph:
x = significantly different from 1% FBS control;
o = significantly different from corresponding salt control.

A: Results for anion exchange HPLC fraction #1. Second subculture cells were used.
B: Results for anion exchange HPLC fraction #2. Second subculture cells were used.
C: Results for anion exchange HPLC fraction #3. Third subculture cells were used.

A


Incorporated 3H-thymidine (cpm)
20000
15000
10000
5000
0
x
x
1 %
10 %
#1' 300ng/ml
control 300
#1' 30 ng/ml
control 30
1 %
10 %
#1' 3ng/ml
control 3
#1' 0.3ng/ml
control 0.3
Treatment
Plate A
Plate B

B


Incorporated 3H-thymidine (cpm)
8000
6000
4000
2000
0
x
x
o
o
o
o
1 %
10 %
#2' 200ng/ml
control 200
#2' 20ng/ml
control 20
1 %
10 %
#2' 2ng/ml
control 2
#2' 0.2ng/ml
control 0.2
Treatment
Plate A
Plate B

C

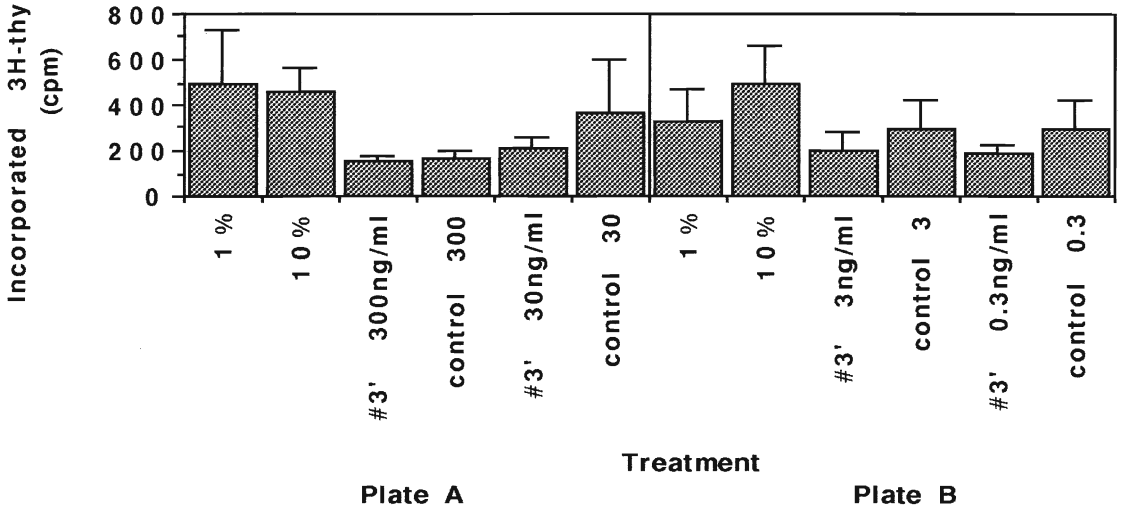
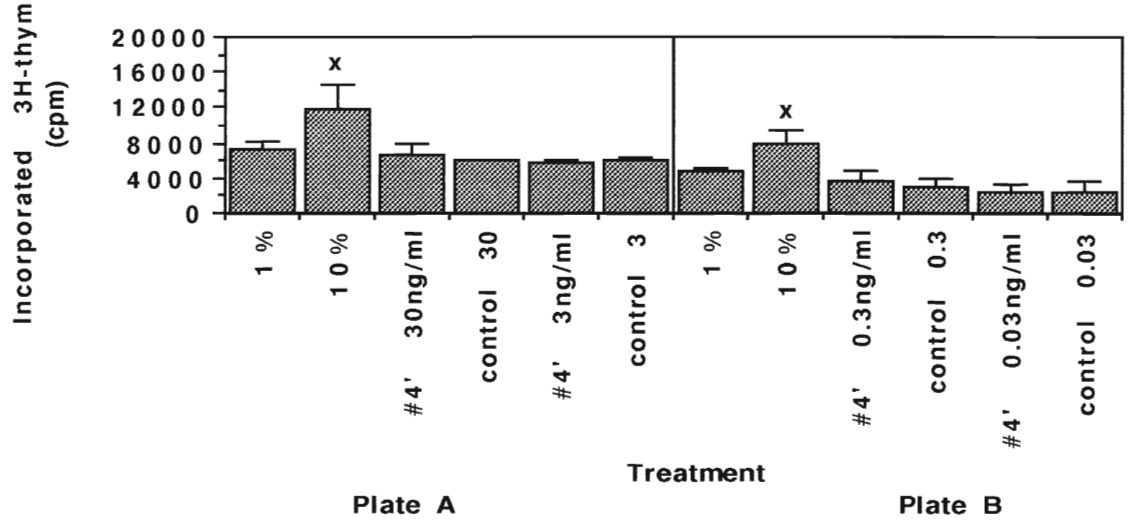

Incorporated 3H-thymidine (cpm)
800
600
400
200
0
1 %
10 %
#3' 300ng/ml
control 300
#3' 30ng/ml
control 30
1 %
10 %
#3' 3ng/ml
control 3
#3' 0.3ng/ml
control 0.3
Treatment
Plate A
Plate B

Figure A3. Effect of Anion Exchange HPLC Fractions #4, 5, and 6 on ^{3}H-thymidine Incorporation in Cultured Chick Embryo Brain Non-neuronal Cells.

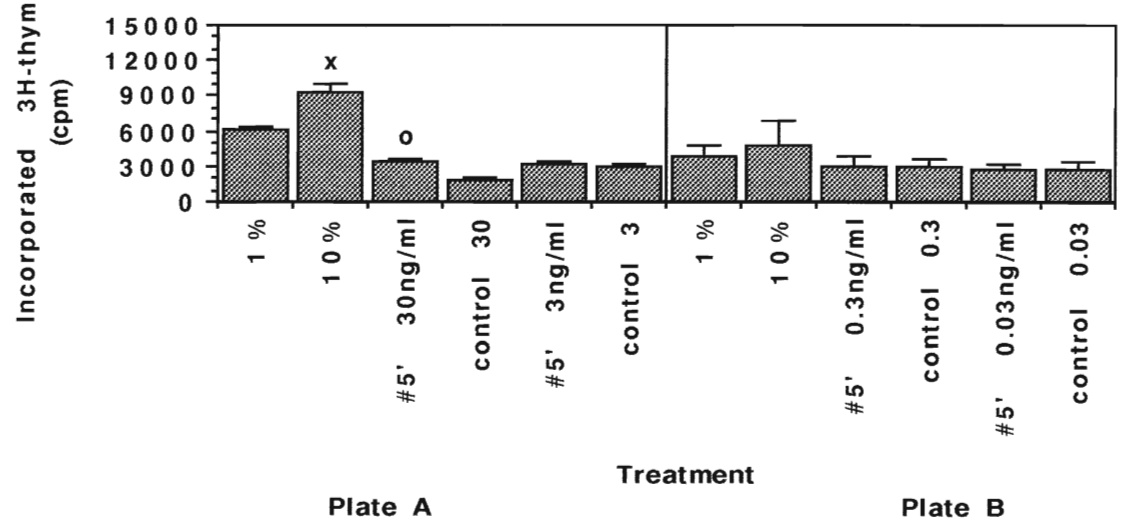
Two twenty-four well plates were assayed per fraction, represented by Plate A and Plate B. Due to the high salt concentration of the anion exchange solutions, a salt control corresponding to the KH_2PO_4 and KCl content of each fraction at every concentration assayed was also tested. Data are presented as mean incorporation of ^{3}H-thymidine in cpm. n = 4 for each treatment. [HPLC = high-performance liquid chromatography; cpm = counts per minute; 1% = 1% fetal bovine serum (FBS); 10% = 10% FBS]
Statistically significant differences ($p \leq 0.05$) are represented by the following symbols over the corresponding bar on the graph:
x = significantly different from 1% FBS control;
o = significantly different from corresponding salt control.

A: Results for anion exchange HPLC fraction #4. Third subculture cells were used.
B: Results for anion exchange HPLC fraction #5. Third subculture cells were used.
C: Results for anion exchange HPLC fraction #6. Fourth subculture cells were used.

A


Incorporated 3H-thymidine (cpm)
20000
16000
12000
8000
4000
0
x
x
1 %
10 %
#4' 30ng/ml
control 30
#4' 3ng/ml
control 3
1 %
10 %
#4' 0.3ng/ml
control 0.3
#4' 0.03ng/ml
control 0.03
Treatment
Plate A
Plate B

B


Incorporated 3H-thymidine (cpm)
15000
12000
9000
6000
3000
0
x
o
1 %
10 %
#5' 30ng/ml
control 30
#5' 3ng/ml
control 3
1 %
10 %
#5' 0.3ng/ml
control 0.3
#5' 0.03ng/ml
control 0.03
Treatment
Plate A
Plate B

C

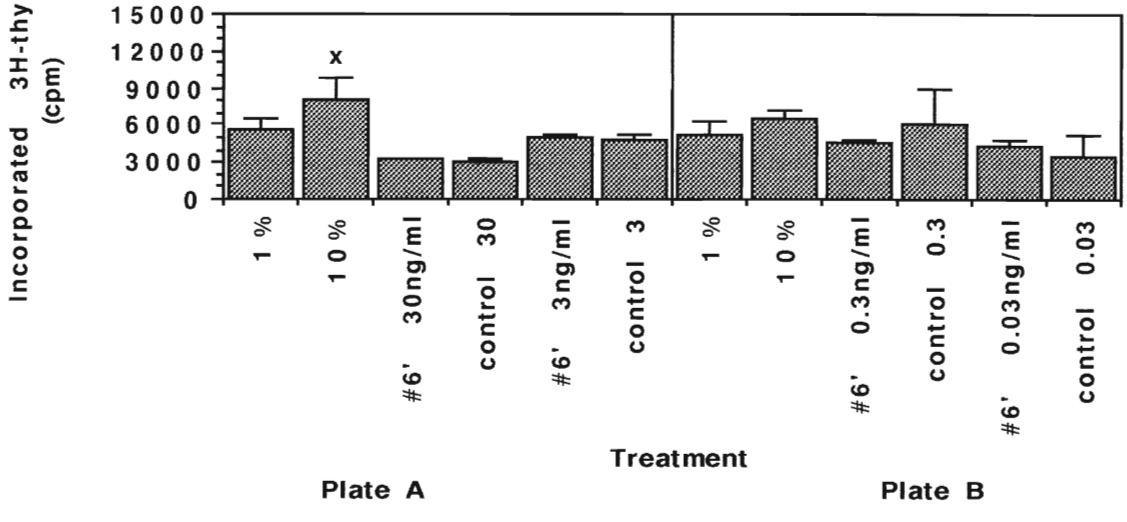
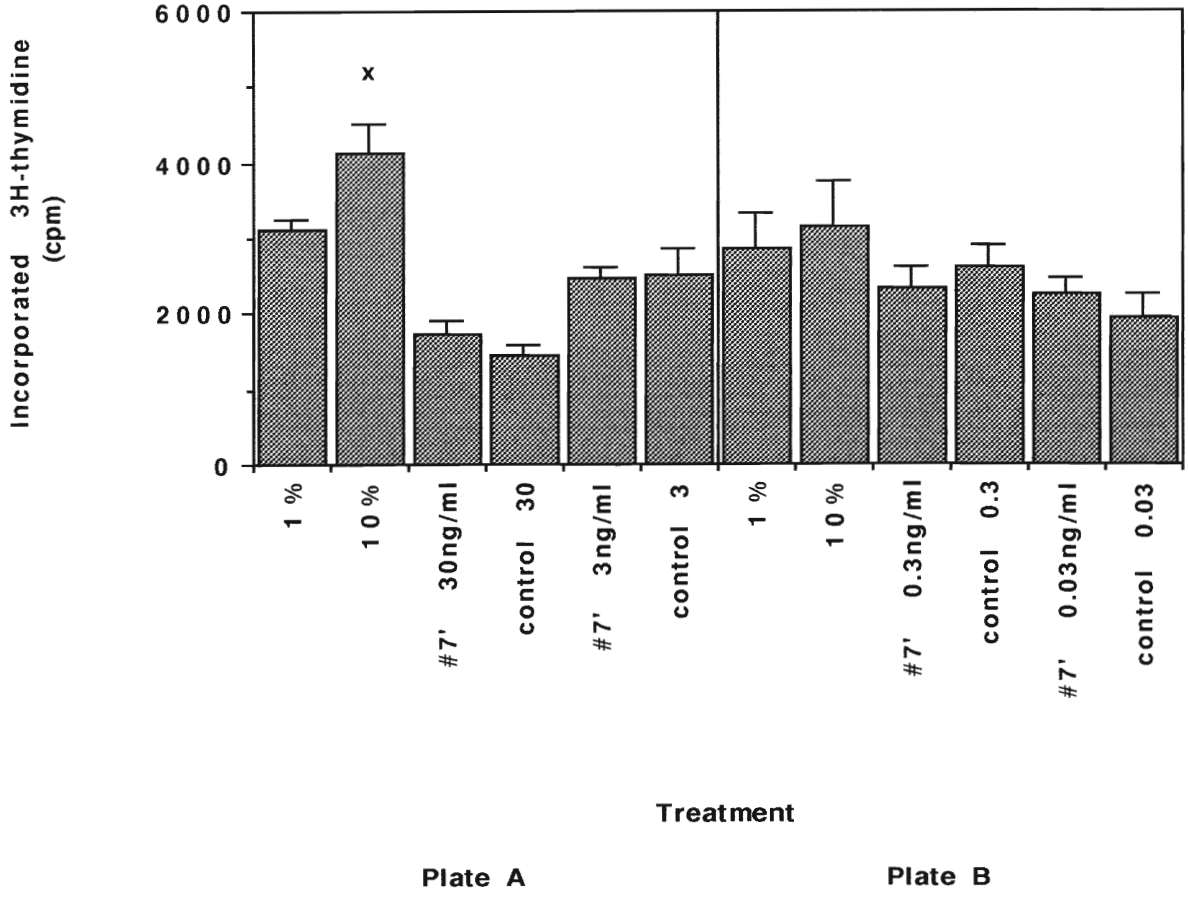

Incorporated 3H-thymidine (cpm)
15000
12000
9000
6000
3000
0
x
1 %
10 %
#6' 30ng/ml
control 30
#6' 3ng/ml
control 3
1 %
10 %
#6' 0.3ng/ml
control 0.3
#6' 0.03ng/ml
control 0.03
Treatment
Plate A
Plate B

Figure A4. Effect of Anion Exchange HPLC Fraction #7 on ^{3}H-thymidine Incorporation in Cultured Chick Embryo Brain Non-neuronal Cells.

Two twenty-four well plates were assayed, represented by Plate A and Plate B. Due to the high salt concentration of the anion exchange solutions, a salt control corresponding to the KH_2PO_4 and KCl content of this fraction at every concentration assayed was also tested. Data are presented as mean incorporation of ^{3}H-thymidine in cpm. n = 4 for each treatment. Fourth subculture cells were used. [HPLC = high-performance liquid chromatography; cpm = counts per minute; 1% = 1% fetal bovine serum (FBS); 10% = 10% FBS] Statistically significant differences ($p \leq 0.05$) are represented by the following symbol over the corresponding bar on the graph:
x = significantly different from 1% FBS control.


Incorporated 3H-thymidine (cpm)
6000
4000
2000
0
x
1 %
10 %
#7' 30ng/ml
control 30
#7' 3ng/ml
control 3
1 %
10 %
#7' 0.3ng/ml
control 0.3
#7' 0.03ng/ml
control 0.03
Treatment
Plate A
Plate B

APPENDIX B

Table BI. Raw Data for Bioassays Giving 10% FBS and 1% FBS Results (Mean ± Standard Deviation)

Figure Number	Plate	10% FBS	1% FBS
1	A	6068.7 ± 657.1	1180.2 ± 36.5
	B	3247.5 ± 935.7	641.1 ± 77.5
6		7597.3 ± 1340.6	3498.0 ± 654.7
7	A	11544.4 ± 970.44	6387.6 ± 336.3
	B	11480.2 ± 1004.1	6075.9 ± 1520.0
9	A	16223.6 ± 1311.8	9827.0 ± 1823.8
	B	13529.3 ± 2416.7	6235.7 ± 345.4
10	A	13431.0 ± 3134.8	4922.7 ± 395.3
	B	10473.2 ± 610.0	4900.3 ± 304.9
11		6783.5 ± 447.2	3971.8 ± 400.0
14	A	7920.2 ± 236.8	3625.6 ± 754.9
	B	4688.1 ± 574.0	2823.0 ± 99.08
	C	6774.4 ± 839.3	3685.1 ± 378.4
15	A	8361.7 ± 576.0	4255.9 ± 213.98
	B	8988.9 ± 838.5	5783.7 ± 764.9
	C	7729.4 ± 1139.6	5312.8 ± 683.7
16	A	6031.7 ± 1391.0	3756.0 ± 388.0
	B	8306.6 ± 224.2	5362.7 ± 2253.6
	C	7717.0 ± 518.3	5808.1 ± 231.3
17	A	6875.7 ± 2373.9	5816.6 ± 442.2
	B	6977.3 ± 1118.8	4330.4 ± 1183.8
24	A	8564.8 ± 398.7	4410.6 ± 331.9
	B	7943.3 ± 1353.5	5023.6 ± 142.2
25		4717.8 ± 823.8	2019.1 ± 163.7
26	A	5987.3 ± 1283.2	1932.5 ± 38.90
	B	7561.8 ± 792.8	3318.9 ± 314.4
27		3940.0 ± 1221.2	1429.3 ± 214.5
28		9767.7 ± 658.3	5045.1 ± 477.6